Physical and Functional Interactions of Human DNA Polymerase α-primase and Replication Protein A

Dissertation

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Tag der öffentlichen Verteidigung.....

Abbreviations

aa	Amino acids
APS	Ammonium per sulphate
ATM	Ataxia telangiectasia protein M
ATP	Adenosine triphosphate
BER	Base excision repair
bp	Base pair
BSA	Bovine serum albumin
°C	Degree celsius
CdK	Cyclin-dependent kinase
CTp58	C-terminus of p58
Da	Dalton
DDK	Dbf-dependent kinase
DNA	Deoxyribonucleic acid
ds	Double-stranded
EDTA	Ethylenediamine tetraacetic acid
EMSA	Electrophoretic mobility shift assay
Fe	Iron
g	Gram
hr.	Hour
hrs.	Hours
HRP	Horse-radish-peroxidase
IB	Immuno blotting (Western blotting)
IP	Immunoprecipitation
k	Kilo
l	Liter
m	Milli
M	Molar

MCM	Mini-chromosome maintenance
mg	Milli gram
min	Minute
ml	Milli liter
mM	Milli molar
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NER	Nucleotide excision repair
Ni	Nickel
NLS	Nuclear localization signals
NMR	Nuclear magnetic resonance
NP-40	Nonidet P-40
oligo	Oligonucleotide
ORC	Origin recognition complex
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCNA	Proliferation cell nuclear antigen
PMSF	Phenylmethylsulfonylfluoride
PNK	Polynucleotide kinase
Pol	Polymerase
Pol-prim	DNA polymerase α-primase
PP2A	Protein phosphatase 2A
Pu	Purine
PVDF	Polyvinylidene fluoride
Py	Pyrimidine
RFC	Replication factor C
RNA	Ribonucleic acid
RPA	Replication protein A
SDS	Natrium dodecyl sulfate

sec	Second
ss	Single-stranded
ssDNA	Single-stranded DNA
SV40	Simian virus 40
TBE	Tris-borate-EDTA
TE	Tris-EDTA solution
TEMED	N,N.N´,N´-Tetramethylethylenediamine
Tris	Tris-(hydroxymethyl)-aminomethane
w/o	Without
wt	Wild type
XP	Xeroderma Pigmentosum
XPA	Xeroderma pigmentosum, complementation group A
XPF	Xeroderma pigmentosum, complementation group F
XPG	Xeroderma pigmentosum, complementation group G

1 Introduction

1.1 Cell cycle and DNA replication

Any living cell and organism is faced with the tremendous task of keeping its genome intact (Alberts et al., 2002). In order to develop in an organized manner and to function in a complex environment, cells are required to duplicate the genetic information prior to cell division. DNA synthesis is also needed during DNA repair processes like nucleotide excision repair (NER), base excision repair (BER) as well as DNA recombination and bypassing lesions when the DNA has been damaged (Hubscher et al., 2002).

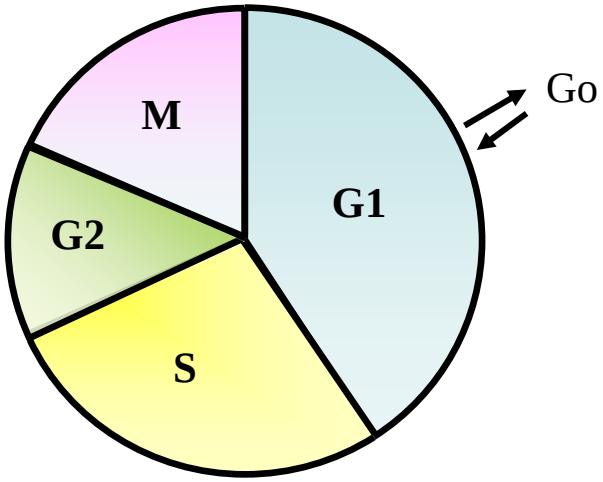

Figure 1.1: Eukaryotic cell cycle

The mitotic cell cycle of eukaryotic cells is divided into four main stages: G1, S, G2 and M (Alberts et al., 2002). After the cell division is finished, the first phase, G1 (G stands for gap) begins. During G1 phase, cells prepare themselves for S phase (S stands for synthesis). Alternatively, cells can reversibly leave the cell cycle into a 'resting stage' called Go. In S phase, chromosomal DNA is duplicated. Before entering mitotic phase (M phase), cells enter into another

growth phase called G2. During mitotic phase (M phase) the whole cellular contents including the genetic information divide into two smaller, identical daughter cells. Faithful duplication of chromosomal DNA is an essential prerequisite for cell division (Conaway and Lehman, 1982) and many regulatory mechanisms monitor progression of the cell cycle. Genome must be replicated once per cell cycle and this process is mainly controlled at the initiation step of the eukaryotic DNA replication (Kozu et al., 1982; Riedel et al., 1982; Shioda et al., 1982; Yagura et al., 1982).

To ensure the efficient and precise duplication of large genome of eukaryotic cells, DNA replication initiates from multiple replication origins distributed along the individual chromosomes (Kelly and Brown, 2000). Activation of replication origins requires the association of the origin recognition complex (ORC), which is a six subunit protein complex and is conserved from yeast to humans. In budding yeast ORC remain bound to origins throughout the cell cycle, whereas in multi-cellular eukaryotes some specific ORC subunits are only bound to chromatin in parts of the cell cycle. Moreover, there are conflicting reports whether ORC remains bound to chromosomes during mitosis (Tanaka et al., 1997).

At the beginning of G1 phase, Cdc6 and Cdt1 are recruited to the chromatin in a ORC-dependent manner. Cdc6, Cdt1 and ORC together load six closely related proteins known as the MCM2-7 complex onto chromatin to form the so-called pre-replicative complex (pre-RC). In organisms from yeast to humans, about 20 copies of the MCM2-7 complex per origin are loaded into chromatin (Kelly and Brown, 2000). The loading of the MCM2-7 complex is necessary for the initiation of DNA replication and is also known as 'licensing' of cells for DNA replication. The MCM2-7 is required throughout S phase for both initiation and elongation, while a sub-complex consisting of MCM4, 6 and 7 has been shown to act as DNA helicase. Replication origins that are no longer occupied by the MCM complex are inactive, and this period of inactivation persists into the next G1 phase, in which a new cycle of activation begins with the recruitment of the

MCM2-7 complex. MCM2-7 complex binds to Cdc45 as well as another replication initiation factor, MCM10, which is required for chromosomal DNA replication and stable plasmid maintenance in *S. cerevisiae* (Merchant et al., 1997). In *S. cerevisiae*, MCM10 is a component of the pre-RC and is required for the association of the MCM complex with origin DNA (Homesley et al., 2000). In *S. cerevisiae* and in *S. pombe*, MCM10 is constitutively chromatin bound (Gregan et al., 2003; Homesley et al., 2000). Another MCM family member, MCM8 contains the typical MCM domain and functional motifs of the MCM2-7 proteins, and recently it is reported that hMCM8 is a crucial component of the pre-RC and that the interaction between hMCM8 and hCdc6 is required for pre-RC assembly (Volkening and Hoffmann, 2005).

The full process of origin "firing" is still not fully understood. There are some reports that chromosomal context is a critical determinant because origins near telomere tend to activate late in S phase and early firing origins moved to a sub-telomeric location become late-firing. Evidence from yeast and mammalian cells indicate that the temporal program of origin activation is established early in the cell cycle, well before DNA replication begins (Raghuraman et al., 1997).

Many eukaryotic cells enter quiescence from G1 phase and pre-RCs are lost (budding yeast). While quiescent mammalian cells are also unlicensed and it is not clear whether such cells loose pre-RCs or they do not assemble them in the first place. Data suggest that in extended Go, MCM proteins are lost (Endl et al., 2001). The next step is the maturation of pre-RC into a pre-initiation complex (pre-IC) by recruitment of the other proteins including Cdc45 and Sld3 (Bell and Dutta, 2002; Blow, 2001; Nasheuer et al., 2002).

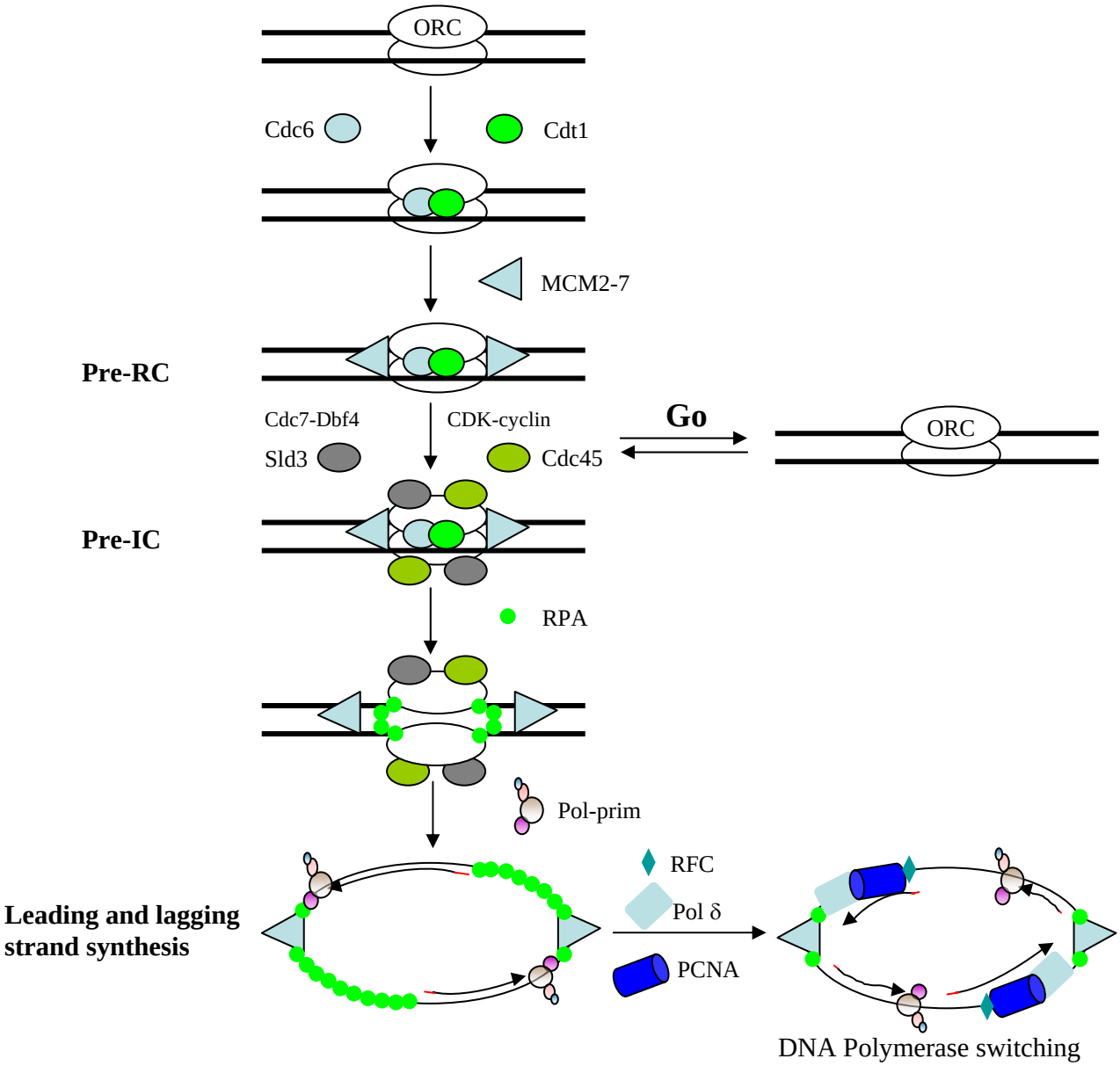

Figure 1.2: The chromosome replication cycle. Schematic diagram of the assembly of replication machinery at ORC, showing proteins responsible for maintaining the separation of the parental strands and unwinding the double helix ahead of the advancing replication fork. At the end of the mitosis Cdc6 and Cdt1 load separately to ORC. Next MCM2-7 proteins are recruited to chromatin by ORC. This pre-RC matures into pre-IC by association of Cdc45 and Sld3. DNA synthesis on both leading and lagging strand need at least two DNA polymerases α and DNA polymerases δ or DNA polymerase ε. RFC loads PCNA ring onto the DNA primer, which binds to the DNA polymerase and acts as a 'sliding clamp' preventing the DNA polymerase from falling off the DNA.

The presence of Cdc45 is required for the assembly of various components of the DNA synthetic machinery at the replication fork including RPA, PCNA, Pol α and Pol ε (Aparicio et al., 1999; Mimura et al., 2000; Walter and Newport, 2000; Zou and Stillman, 2000). Although the exact order of assembly of these components is unclear, studies in *S. cerevisiae* and *Xenopus* egg extracts agree that RPA is required for the loading of Pol α (Mimura et al., 2000; Tanaka and Nasmyth, 1998; Walter and Newport, 2000; Zou and Stillman, 2000). However, in *Xenopus* egg extracts, neither RPA nor Pol α were required for the association of DNA Pol ε with chromatin (Izumi et al., 2000).

Initiation of DNA replication is triggered by the cooperative action of at least two sets of protein kinases, cyclin-dependent kinases (CDKs) and Dbf4-Cdc7, which recruit Cdc45 to the origin of DNA replication (Zou and Stillman, 1998). In all organisms examined, S phase is normally activated by a specific cyclin CDK-combination. In metazoans, CDK2 acts as the S phase CDK together with cyclin A and E. CDKs play a second critical role in regulating DNA replication: they inhibit the assembly of new pre-RCs. This prevents re-initiation from origins that get activated during S phase (Voitenleitner et al., 1999). CDKs have also been reported to physically associate with multiple components of the pre-RC. In *X. laevis*, it is clear that CDKs are associated with chromatin in a reaction that depends on ORC and Cdc6, making it likely, that CDKs are present at origins (Furstenthal et al., 2001). There is substantial evidence that human Cdc6 co-immunoprecipitates with CDKs and this interaction has also been recapitulated with purified recombinant proteins *in vitro* (Furstenthal et al., 2001; Jiang et al., 1999; Petersen et al., 1999).

At this stage, after RPA and Cdc45 are already associated with the chromatin, Pol α is loaded. There is good evidence that Cdc7 stimulates initiation by phosphorylating MCM2 (Sclafani, 2000). DNA polymerases are unable to initiate chain synthesis *de novo*, therefore 'primase' synthesizes a short tract of ribonucleotides, which then act as a 'primer' for DNA synthesis. Continuous DNA

synthesis requires a DNA polymerase that can synthesize long stretches of DNA in a processive manner (i.e. without falling off). Pol α is unsuitable for this job because it exhibits very low processivity ~30 nucleotides per initiation. In addition, Pol α does not have the 3' to 5' exonuclease activity for proof-reading (Kunkel et al., 1991). Therefore, DNA synthesis requires a 'DNA-polymerase switch' in which a more processive, high fidelity DNA polymerase replaces Pol α. *In vitro* studies showed that two DNA polymerases, Pol α and Pol δ are sufficient for complete, efficient SV40 DNA replication. Strong evidence that a third DNA polymerase, Pol ε, is required for efficient chromosomal DNA replication has come from work in yeast and *xenopus* (Waga et al., 2001).

Highly processive DNA synthesis by the Pol δ and Pol ε requires an auxiliary factor called PCNA (proliferating cell nuclear antigen). PCNA is a homotrimer that forms a ring shaped structure. This ring encircles the template DNA and is thus topologically linked to the DNA. PCNA binds to the DNA polymerase and acts as a ‘sliding clamp’, preventing the DNA polymerase from falling off the DNA. The PCNA ring must be loaded onto the DNA primer end and this is accomplished by RFC (replication factor C), also known as the ‘clamp loader’ (Mossi et al., 2000).

1.2 DNA polymerase α-primase

DNA polymerase α (Pol α) holds a special position among the growing family of eukaryotic DNA polymerases. In fact, Pol α is associated with primase to form a four subunit complex and, as a consequence, is the only enzyme able to start DNA synthesis *de novo*. Because of this peculiarity it plays an essential role in the initiation of DNA replication at chromosomal origins and in the discontinuous synthesis of Okazaki fragments on the lagging strand of the replication fork. In addition to its enzyme activities during DNA replication, DNA polymerase α-primase appears to play a key role in coordinating DNA replication, DNA repair, and cell cycle check points (Foiani et al., 1997; Holmes and Haber, 1999).

Pol α was the first DNA polymerase identified in 1957 and for many years people believed that Pol α was the only DNA polymerase required for chromosomal DNA replication. Later with the discovery of DNA polymerases β and γ (Pol β and Pol γ respectively) in early 1970s, people suggested that Pol α is the enzyme responsible for nuclear DNA replication, Pol β is involved in DNA repair and Pol γ replicates mitochondrial DNA. All known pols lack the capacity to start DNA chains *de novo* on single-stranded (ss) templates and require the 3'-end of a pre-existing primer molecule. Several strategies evolved during evolution to solve this problem with the most frequent strategy of viruses, prokaryotes, and eukaryotes being the synthesis of a short RNA molecule synthesized by DNA primase that can be considered as a specialized RNA polymerase required for initiation of DNA chains (Arezi and Kuchta, 2000; Foiani et al., 1997).

1.2.1 Structure of pol-prim complex

In all eukaryotic organisms analyzed to date, DNA polymerase α-primase is a four subunit complex p180, p68, p58 and p48 (Figure 1.3) evolutionary conserved from yeast to human. Each subunit is essential for cell viability in yeast (Arezi and Kuchta, 2000; Foiani et al., 1997). The largest, 180 kDa, polypeptide (p180) shows DNA polymerase activity, whereas the p48 subunit contains the catalytic center of the RNA polymerase (primase) activity (Nasheuer and Grosse, 1988; Santocanale et al., 1993).

The largest subunit, p180, contains seven sequence motifs conserved between Pol α homologs (Figure 1.4). The most highly conserved region, I, contains two aspartate residues, which are probably involved in the binding of Mg^{2+}, whereas the second most conserved region II, probably interacts with the incoming dNTP and with the template primer (Copeland and Wang, 1993b). No enzymatic activity has been found to be associated with the second largest subunit, also called the B subunit. The apparent molecular mass of the B subunit is in the range of ~70

kDa in mammalian cells to ~90 kDa in budding yeast (Foiani et al., 1994; Hubscher et al., 2002).

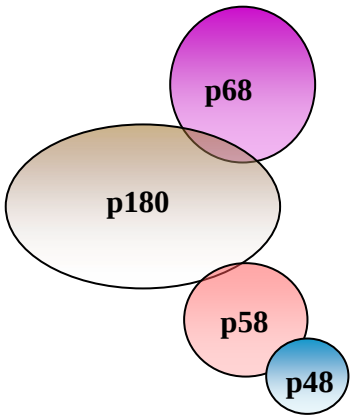

Figure 1.3: Schematic model of the DNA polymerase α-primase. The largest 180 kDa, polypeptide (p180) contains DNA polymerase activity, p58 and p48 comprise primase and p68 is the fourth, tightly bound subunit.

The p58 polypeptide forms a tightly bound sub-complex with p48 and is generally considered as the second primase subunit, even though its physiological role is still poorly defined. It has been suggested that (1) p58 helps to stabilize the thermolabile nature of the catalytic activity of p48 (Santocanale et al., 1993; Schneider et al., 1998). (2) p58 is required for productive interaction between p48 and p180 (Longhese et al., 1993) possibly playing an essential role in the transition between RNA primers synthesis and their subsequent elongation by Pol α (3) p58 contains a nuclear localization signal that appears to be required for translocation of p48 within the nucleus through a piggy-back binding transport mechanism (Mizuno et al., 1996).

Comparison of the amino acid sequences of the small and large subunit of eukaryotic primases reveals few conserved motifs (Copeland, 1997; Prussak et al., 1989; Stadlbauer et al., 1994). All conserved charged residues appear necessary for efficient primer synthesis, in particular E105, D109 and D111 in

motif IV (Copeland and Tan, 1995). The two conserved aspartates (D109 and D111) in region I of human p48 together with aspartate D306 in region II are involved in metal ion binding (Augustin et al., 2001). In addition, region II contains an arginine at position 304, which probably contacts the synthesized primer. The neighboring, least conserved region A controls species specificity of mouse polymavirus DNA replication *in vitro* (Augustin et al., 2001; Kautz et al., 2001; Kirk and Kuchta, 1999).

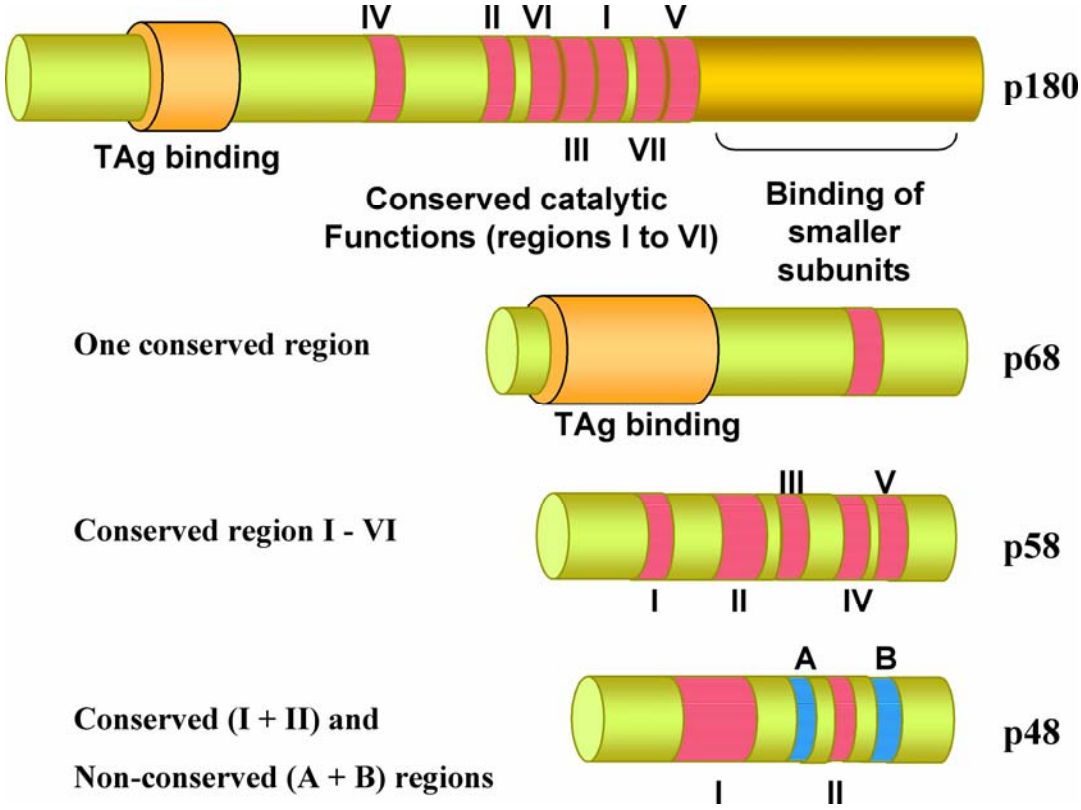

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Figure 1.4: Subunit structure of DNA polymerase α-primase. Each subunit contains regions, which are conserved from yeast to humans, marked in roman numerals. SV40 DNA replication requires a single virus-encoded protein, the SV40 large tumor antigen (T antigen), which functions both as an initiator protein by binding to the SV40 origin and as a DNA helicase at the replication fork. The functional equivalents of TAg have not been identified in eukaryotic cells. The presence of TAg stimulates primer synthesis by Pol α-primase.

1.2.2 Reaction mechanism of pol-prim complex at the replication fork

After pol-prim becomes associated with the initiation complex at origins of DNA replication, the primase subunit starts to synthesize a short RNA primer 7-10 nucleotides long, which are elongated by a stretch of 20 to 30 deoxyribonucleotides by the large subunit of pol-prim (Hurwitz et al., 1990; Waga and Stillman, 1998). A similar sequence of reactions takes place for synthesis of Okazaki fragments on the lagging strand. After the synthesis of this RNA-DNA hybrid molecule (also called initiator DNA) the pol-prim complex is substituted by Pol δ (Matsumoto et al., 1990; Tsurimoto and Stillman, 1991) and perhaps Pol ε (Waga et al., 2001), which continue the processive synthesis of the leading strand (Foiani et al., 1997). The synthesis of the Okazaki fragments to their final length of approximately 200 nucleotides also requires the switch from pol-prim to Pol δ or Pol ε (Hubscher et al., 2002; Kelly and Brown, 2000).

Pol-prim catalyzes the synthesis of RNA-DNA primers in a minimum of five subsequent steps: template binding, NTP binding, dinucleotide formation, extension to a functional RNA primer, and primer transfer to the Pol α catalytic site for elongation. Pol-prim binds to ss-DNA and can slide on it before NTP binding, suggesting that the enzyme does not need to synthesize a primer at the site where it first binds the template DNA (Kirk et al., 1997). On a ss-DNA template pol-prim shows a strong preference for deoxypyrimidine polymers with a minimum chain length of 5-10 residues (Badaracco et al., 1986). On templates containing a mixture of purines and pyrimidines, pol-prim does not initiate synthesis randomly, but prefers certain sites (Yamaguchi et al., 1985). After binding the template, the primase subunit next must bind two NTPs to catalyze the formation of a dinucleotide. Sheaff and Kuchta showed that dinucleotide formation appears to be the rate-limiting step, as dinucleotides synthesize at a rate of 0.003 s^{-1} and then rapidly extend to full-length RNA primers of 7-10 nucleotides (Sheaff and Kuchta, 1993). Later, it was seen that NTPs concentration also influences the selection of initiation sites. At low NTPs concentrations pol-prim is free to slide along the DNA until it comes to a

preferred pyrimidine-rich sequence, while at high NTPs concentration pol-prim initiates synthesis at the first potential site found after DNA binding (Kirk et al., 1997). Pol-prim also counts the length of the RNA primer and in the absence of the dNTPs, pol-prim continues to synthesize RNA primers up to 40-50 nt (Badaracco et al., 1985; Sheaff and Kuchta, 1993).

Photo-cross linking studies of single-stranded DNA-primase complexes (Arezi et al., 1999) revealed that the isolated p48 and p58 subunits both reacted with DNA upon photolysis, but when photolysis was performed using the p48/p58 primase complex, only the p58 subunit reacted with the DNA. After primer synthesis by the complex, again p58 is the only subunit that reacts with the DNA (Arezi et al., 1999). Pol-prim must first bind a DNA template before synthesizing RNA primers (Frick and Richardson, 1999; Thompson et al., 1995). The eukaryotic pol-prim complexes bind DNA with a K_D of 0.1 to 1.0 μM and protect 9 nucleotide of the primer and 13 nucleotides of the template from nuclease digestion (Anarbaev et al., 1995; Thompson et al., 1995). After the generation of a unit-length RNA primer, subsequent primase activity stops until Pol α elongates primer or primer dissociates from primase. Unit-length primer acts as a termination signal to the primase, whereas pol-prim does not start synthesizing DNA, from the primers which are smaller than 7 nucleotides (Sheaff and Kuchta, 1993).

1.2.3 Regulation of pol-prim during the cell cycle

Because all cells do not continuously divide, pol-prim activity must be tightly regulated. Therefore, understanding the role that pol-prim plays in cell division and proliferation can lead to improved cancer therapies. The expression of human pol-prim genes is up-regulated at the level of transcription when cells leave a quiescent state to enter the mitotic cell cycle, but the amount of pol-prim transcript does not fluctuate once mammalian cells are actively cycling (Pearson et al., 1991). In mouse FM3A cells, mRNA levels for the p180 and p70 subunits vary slightly throughout the cell cycle (Miyazawa et al., 1993). In budding yeast,

the genes coding for the pol-prim subunits are periodically transcribed at the G1/S boundary, together with several other DNA synthesis genes (Foiani et al., 1989; Johnston et al., 1990). The genes encoding human Pol α and the two primase subunits are down-regulated during cell differentiation (Moore and Wang, 1994). However, the pol-prim subunits are very stable proteins whose level is not rate limiting for DNA synthesis, since yeast cells can undergo several cell divisions, even when the level of these polypeptides drops well below the physiological amount (Falconi et al., 1993).

Mammalian and yeast pol-prim complexes are known to be phosphorylated in a cell cycle-dependent manner (Foiani et al., 1995; Nasheuer et al., 1991). The p180 and p68 subunits in human cells at G2/M, the fission yeast p180 subunit in late S, and the p68 homolog in budding yeast at G1/S are cell cycle-dependently phosphorylated (Dehde et al., 2001; Foiani et al., 1997; Schub et al., 2001). Phosphorylation of the p58 and p48 subunits was also observed, but it seems not to vary during the cell cycle (Foiani et al., 1995; Nasheuer et al., 1991). Phosphopeptide maps of human p180 and p68 indicate that a CDK is most likely responsible for the modification (Foiani et al., 1997; Nasheuer et al., 1991; Voitenleitner et al., 1997). Recently, *in vitro* studies showed that p180 is also a substrate of DDK (Weinreich and Stillman, 1999). In human cells two immunologically distinct subpopulations exist which are differentially phosphorylated (Dehde et al., 2001). The hypo-phosphorylated pol-prim is present in the nucleus from G1 to early S phase, but does not co-localize with the incorporated nucleotide analog BrdU, whereas the localization of the hyper-phosphorylated form overlaps with newly synthesized DNA in the nucleus (Dehde et al., 2001). The phosphorylation of pol-prim by cyclin A-CDK2, but not by cyclin E-CDK2 prevents its interaction with the viral initiator SV40 T antigen and abolishes origin-dependent initiation (Dehde et al., 2001; Schub et al., 2001; Voitenleitner et al., 1997). The inhibitory effect of CDKs is counteracted by PP2A or by mutating potential CDK phosphorylation sites of p180 and p68 (Dehde et al., 2001; Schub et al., 2001; Voitenleitner et al., 1999).

Primase regulation is also linked to the phosphorylation of histone H1, which in its dephosphorylated form inhibits primer synthesis in a dose-dependent manner and its phosphorylation relieves some of this inhibition (Takada et al., 1989; Takada et al., 1994). Retinoblastoma protein is also proposed to be a regulator of human primase activity. It exists in both phosphorylated and dephosphorylated states. The phosphorylated form binds to the Pol α/primase complex and stimulates primase activity significantly more than the dephosphorylated form (Takemura et al., 1997).

1.3 Replication protein A

Replication protein A (RPA) is a heterotrimer protein, consisting of three subunits of 70, 32 and 14 kDa (p70, p32 and p14, respectively) (Iftode et al., 1999). Homologous heterotrimeric ssDNA-binding proteins have been identified in all eukaryotes examined (Atrazhev et al., 1992; Brill and Stillman, 1989; Nasheuer et al., 1992). RPA was originally purified from human cell extracts as a component essential for simian virus 40 (SV40) DNA replication *in vitro* (Wobbe et al., 1987; Wold and Kelly, 1988). RPA binds with high affinity to single-stranded DNA (ssDNA) (Fairman and Stillman, 1988) and with low affinity to double-stranded DNA (dsDNA) and RNA (Kim et al., 1992; Wold et al., 1989). RPA also functions in DNA repair (Coverley et al., 1992; Coverley et al., 1991) and recombination (Heyer et al., 1990; Moore et al., 1991). RPA becomes phosphorylated in the cells when bound to ssDNA (Din et al., 1990; Henricksen et al., 1994). RPA has been found to interact physically with the replicative OBP (origin binding protein) / helicase large T-antigen of SV40 (Dornreiter et al., 1993; Weisshart et al., 2000), with pol-prim (Braun et al., 1997), with proteins of the nucleotide excision repair (NER) pathway (XPA, XPG, XPF) (He et al., 1995; Wang et al., 2000), with recombination specific proteins (Golub et al., 1998; Park et al., 1996), and with transcriptional activators (GAL4, VP16, p53, RBT1) (Cho et al., 2000; Dutta et al., 1993). The p70 subunit of RPA contains three

functionally distinct domains: an N-terminal domain, a central ssDNA-binding domain, and a C-terminal subunit ssDNA interacting domain which is also required for the association with the smaller subunits (Jacobs et al., 1999). The N-terminal domain contains the interaction domain for pol-prim, which consists of two distinct regions: one (amino acids 1-170) that stimulates Pol α synthetic activity and another (amino acids 170 - 327) that increases Pol α processivity (Braun et al., 1997).

Aim

DNA polymerase α-primase is the only enzyme capable to start DNA synthesis *de novo*. The major role of pol-prim is the initiation of DNA replication at chromosomal origins and in the discontinuous synthesis of Okazaki fragments on the lagging strand of the replication fork. Recently a sequence similarity between the p58 primase subunit and the 8 kDa DNA binding domain of Pol β was reported. Since regions that are highly conserved evolutionary often play important functions, we hypothesized that this Pol β-like sequence in p58 might have similar DNA binding function. Therefore, we investigated whether primase binds DNA. In addition, we were interested to know whether ssDNA, dsDNA or partially ssDNA and dsDNA structure were preferentially recognized.

RPA is a ssDNA-binding protein and is known to interact physically with primase *in vitro*. We were interested to know, whether any co-operation between RPA and primase exists to bind DNA and whether RPA binds to ectopically expressed pol-prim. The distribution and movement of pol-prim in human cells is still not fully understood. Therefore, auto-fluorescent fusion proteins (GFP-fusion) were produced and ectopically expressed to study intercellular movement and distribution of Pol α subunits.

2 Materials and Methods

2.1 Materials

2.1.1 Cell lines

HEp2 cell line: Adherent human epithelial-like Homo sapiens cell line.
Cultured in DMEM medium with 10% FCS. (ATCC = CCL-23).

HEK293: Human Embryonic Kidney cells. Cultured in DMEM medium with 10% FCS. (ATCC = CRL-1573).

2.1.2 Bacterial strains

E. coli HB101 from Dr. H-P. Nasheuer.

E. coli BL21 (DE3) from Dr. H-P. Nasheuer.

2.1.3 DNA (Plasmids, Constructs)

pCMV5T7C-p180 from Dr. Wegner, University of Erlangen.

pCMV5T7C-p68 from Dr. Wegner, University of Erlangen.

øX174ss-DNA, New England Biolabs.

Poly(dT), GE Health previously Amersham Bio-Sciences.

pET-His Hp48 from Dr. H-P. Nasheuer.

pET-His Hp58-Hp48 from Dr. H-P. Nasheuer.

pET11p48-Hisp58CT from Dr. H-P. Nasheuer.

M13-ssDNA from New England Biolabs.

Activated DNA from Dr. H-P. Nasheuer.

pUC19, Cloning vector for *E. coli* (Yanisch-Perron et al., 1985).

pBR/p180 cDNA, contains p180-DNA from Dr. H-P. Nasheuer.

pBluescript®□□KS, Cloning vector for *E. coli*, (Stratagene, Heidelberg).

pFastBac1®, Cloning vector for *E. coli* from Dr. H-P. Nasheuer.

pUC-p68/-Xba/-Sac , pUC-derivative containing p68-DNA
from Dr. H-P. Nasheuer.

2.1.4 Oligonucleotides

All oligonucleotides were purchased from BioTeZ Berlin-Buch GmbH, Germany, except purine and pyrimidine oligonucleotides (oligo pu and olio py) which were from MWG-Biotech AG, Ebersberg, Germany.

Table 1.

Oligonucleotides	Sequence 5' to 3'
(oligo 63)	GTTCAACCAGATATTGAAGCAGAACGCAAA AAGAGAGATGAGATTTAGGCTGGGAAAAGT TAC (phix174 sequence)
PV1-SLD-HHT (oligo 58)	GTATTGCGGTACCCTTGTAGTCGACGGATG TGCTTTCCGTCCTGATGAGTCCGTGAGG
C5A (oligo 36)	AAAAAGAGAGATGAGATTTAGGCTGGGAAA AGTTAC
C1-37 (oligo 37)	GTTCAACCAGATATTGAAGCAGAACGCAAA AAGAGAG
C1-29 (oligo 29)	GTTCAACCAGATATTGAAGCAGAACGCAA
C1-21 (oligo 21)	GTTCAACCAGATATTGAAGCA
Pyrimidine (Py) (oligo Py)	TCGAGGATCCTCTTTCTCCCTTATTCCTCT TTCCCTCTCTTTTCTTTTCCTCCCCTTCTC TTTCTCTAGA TCTGATCGAT
Purine (Pu) (oligo Pu)	ATCGATCAGATCTAGAGAAAGAGAAGGGGA GGAAAAGAAAAGAGAGGGAAAGAGGAATAA GGGAGAAAGAGGATCCTCGA

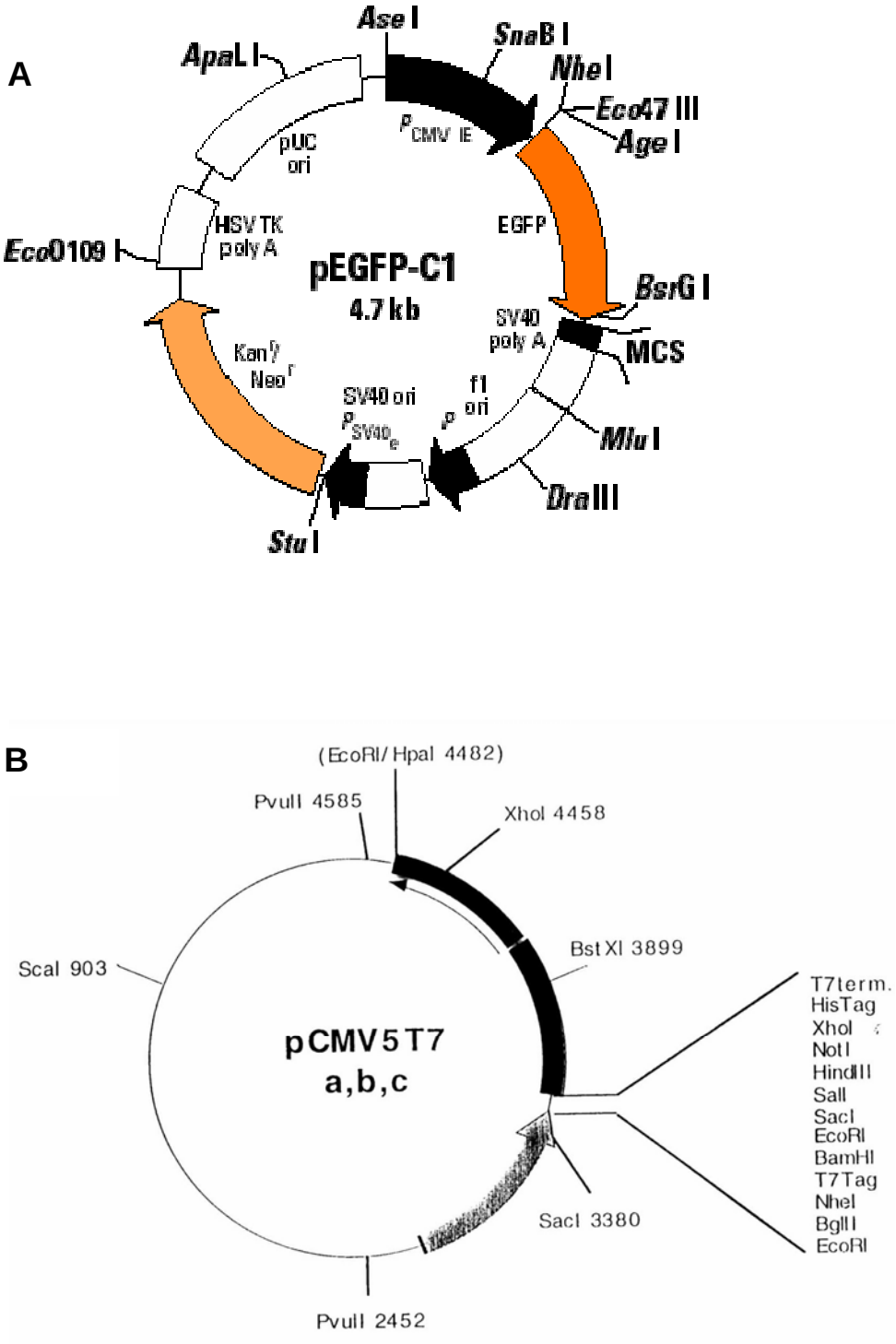

Figure 2.1: The figures above summarize the features of the pEGFP-C1 and pCMV5T7 vectors.

2.1.5 Restriction enzymes

EcoRI, XbaI, ClaI, NcoI, BamHI, NarI, NheI, BglII, EcoRV, NheI, NarI, SalI, NotI, AgeI, SacI, HhaI, Bsp-DI, KasI, NarI, pflmI, BSU36I, AflII, XcmI, KpnI, HindIII, XbaI, PstI, XmnI, BglI, SacII, SphI, HhaI, New England Biolabs (10x-Buffer 1, 2, 3, 4 and 100x-BSA for restriction digest were used).

2.1.6 Proteins

RPA: From Dr. H-P. Nasheuer.

DNA-Polymerase □ (Klenow-Fragment), T4-Polynukleotidkinase (PNK), T4 DNA-Ligase, from New England Biolabs.

p48 : Primase small subunit, from Bernadette Ashe.

2.1.7 Antibodies

Primary antibodies

2CT25: Murine monoclonal antibody anti-DNA polymerase p180 subunit. from Dornreiter, Pette-Institut Hamburg.

p68: Rabbit antiserum against mouse DNA polymerase subunit p68, from Dr. H-P. Nasheuer. Dilution ratio for western blotting, 1: 1000

70B: Murine anti-human RPA p70 monoclonal antibody, from Mark Kenny, University of New York, USA.

KAS2: Rabbit antiserum against primase from Dr. H-P. Nasheuer.

Secondary antibodies:

Alkaline Phosphatase-conjugated goat anti-mouse IgG antibody, Promega Biotech, Heidelberg. Dilution ratio for western blotting, 1: 7,500

Alkaline Phosphatase-conjugated goat anti-rabbit IgG antibody,
Promega Biotech, Heidelberg. Dilution ratio for western blotting 1: 7,500

Horseradish Peroxidase-conjugated goat anti-mouse IgG antibody, Jackson ImmunoResearch Laboratories, Inc. West Grove, USA. Dilution ratio for western blotting, 1: 2,500

2.1.8 Protein-molecular weight marker

Pre-stained marker (from Sigma Aldrich)

205 kDa α_2-Macroglobulin
130 kDa β-Galactosidase
90 kDa Fructose-6-phosphate kinase
64 kDa Pyruvate kinase
53 kDa Fumarase
37 kDa Lactic dehydrogenase
32.6 kDa Triosephosphate isomerase

10 kDa ladder (from Invitrogen)

10-120 kDa in each 10 kDa step, with additional 160 kDa and 220 kDa.

Protein marker (from New England BioLabs)
Broad range (2-212 kDa)

Protein marker (from Gibco /BRL)
10 kDa ladder

2.1.9 Apparatus

Abimed, Düsseldorf, Germany
Gilson Pipetman 2 □l, 20 □l, 200 □l, 1000 □l

Fujifilm Europe, Düsseldorf, Germany
Phosphor imager FLA-5100

Beckman Laboratories, USA
Beckman Centrifuge

Bender and Hohbein, München, Germany
Vortex Genie 2

Biometra ^{R} biomedizinische Analytik, GmbH, Göttingen, Germany
Polyacrylamide gel electrophoresis clips

BioRad Laboratories GmbH, München, Germany
Mini ProteanII^{TM}-Blot apparatus

Carl-Zeiss GmbH, Jena, Germany
Microscope Telaval
Fluorescence microscope, Axiovert S100
Confocal Laser scanning microscope LSM 510

Consort, Wasserburg, Germany
Consort-microcomputer electrophoresis
power supply E 443 and E 752

Eastman Kodak Company Rochester, New York, USA
Kodak Scientific Imaging Filming Biomax ^{TM} MR

Eppendorf Gerätebau, Netheler and Hinz GmbH, Hamburg, Germany
Eppendorf labcentrifuge 5403
Eppendorf Thermomixer 5436

Forma Scientific, Inc., USA
Forma Scientific-Biofreezer (-80°C)

Haake, Karlsruhe, Germany
Waterbath Haake DC1

Hoefer Scientific Instruments, San Francisco, USA
Gel dryer SE 1150

Mat Tek Corporation, 200 Homer Ave. Ashland MA, USA
35 mm Glass Bottom Microwell dishes

Mettler, Bach, Germany
Balance PC 2000

NUAIRE™, USA
CO_2/ water incubator for cell culture

Privileg, Quelle, Nümberg, Germany
Privileg Öko Refrigerator and freezer

Renner GmbH, Darmstadt, Germany
single-used yellow and blue tips
pasteur-pipettes

Sartorius AG, Göttingen, Germany
balance MC1 research RC 210 P

Schott, Jena, Germany
Schott flask, glass beaker

2.1.10 Chemicals

All chemicals if not mentioned are of p.A. quality from Biomol, Hamburg; Fluka-Sigma-Aldrich; E. Merck AG, Darmstadt; Carl Roth GmbH & Co, Karlsruhe, and Serva, Heidelberg.

Sigma-Aldrich

2-Mercaptoethanol, Acrylamide/bis-Acrylamide 30% solution, Ammonium persulphate (APS), Ammonium sulphate, Calcium chloride, Ethanol, Ethidium bromide, Ethylenediamine tetraacetic acid (EDTA), Magnesium chloride tetrahydrate, Glutaraldehyde, Glycerol, Glycine, Imidazole, N-(2-Hydroxyethyl)piperazine-N´-(2-Ethanesulfonic acid) (Hepes), Magnesium chloride, N,N.N´,N´-Tetramethylethylendiamin (TEMED), Sodium chloride, Sodiumdodecyl sulphate(SDS), Phosphate-buffered salt solution (PBS), Triton X-100, Trizma base, Dulbecco´s medium, Bromophenol blue, Glycerol, Iron sulfate heptahydrate, Iron nitrate nonahydrate, 2,2'- Dipyridyl, DNaseI.

Qiagen, Germany	All plasmid purification kits
Biotez, Germany	Oligonucleotides
Roche Biochemicals, Mannheim, Germany	Complete protease inhibitors
Invitrogen, Germany	Penicillin/Streptomycin, Phenol
Neomarkers, Germany	Actin antibody
Biochrom KG, Berlin, Germany	Fetal Calfserum (FCS)
BioRad Laboratories GmbH, München, Germany	BioRad Protein Assay for Bradford
Gerbu, Gaiberg, Germany	NBT, BCIP
Whatman Limited, Maidstone, England	Whatman 3mm paper
Ge Health previously Amersham Bioscences, UK	Protein G Sepharose
NEN™ Life Science Products, Inc. USA	PolyScreen PVDF Transfer Membrane
Novagen, Germany	T7-tag antibody agarose
Sigma Chemical CO. St. Louis USA	Bovine Albumin (BSA), Thymidine
MP Biomedicals, formely ICN Biomedicals, Inc. USA	α-ATP
MP Biomedicals, formely ICN Biomedicals, Inc. USA	γ-ATP

2.1.11 Computer programs

Program	Manufacturer	Application
Word®	Microsoft®	Text
Power point X for Mac®	Microsoft®	Diagrams
DNA Strider®	C. Marck, CEA	Restriction Maps
EndNote Plus®	Thomson ISI ResearchSoft	Literature survey
Image Gauge V4.21	Fuji Photo Film Co Ltd.	Quantification of Phosphorimager images
Adobe®Photoshop® 7.0	Adobe	Scanning of autoradiograms, gels, Western blots

2.2 Methods

2.2.1 Cell culture

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium. FCS (10%) was added to provide growth factors. Cells were grown in a CO_2 incubator (NuAireTM IR autoflow, Minnesota, USA) at 37°C and 10% CO_2 with 95% humidity.

2.2.2 Preparation of crude cell extracts

Adherent cells were detached with 0.25% trypsin and 1% EDTA/PBS and centrifuged at 240 x g for 5 min. After two times washing with PBS, the pellet was incubated in lysis buffer (0.15 M TBS, pH 7.4, 100 μg/ml PMSF, 1 μg/ml Aprotinin, 1 μg/ml Leupeptin, 1% NP40) on ice for 30 min. Debris and nuclear structures were removed by 10,000 x g at 4°C for 10 min. Protein concentration was measured from the lysates using the Bradford method (See below).

2.2.3 Determination of protein concentration

Protein concentration was determined by Bradford assay (Bradford, 1976). BSA was serially diluted and used as a standard. 1.0 ml diluted dye reagent (1:5 in water) was added to each sample tube as well as to the standards and mixed several times by gentle inversion. Sample buffer was used as a negative control. After 15 min at room temperature, OD_{595} values versus negative control were measured. OD_{595} versus concentration of standards was plotted. Protein of interest was calculated from the standard curve using Microsoft Excel Program.

2.2.4 Immunoprecipitation

HEK 293 cells were lysed and protein concentration was measured by Bradford method (section 2.2.3). 500 μg of protein was incubated with T7-tag antibody

agarose beads (Novagen) for 2-3 hrs. at 4°C on a head over bottom mixer. Afterwards beads were washed twice with wash buffer (TBS pH 7.4, 0.25% NP-40, 100 μg/ml PMSF and 1 μg/ml Leupeptin) and boiled for 5 min. prior to loading on SDS gel.

2.2.4.1 Co-immunoprecipitation

Cellular proteins are often associated with various proteins. Some of these complexes are very stable and can be detected after cell lysis. By precipitating one protein, the other subunit of the complex can be co-precipitated and these protein-antibody complexes can be analyzed by western blotting using antibodies recognizing other protein components in the complex. To investigate proteins binding to recombinant Polymerase α-primase subunits, crude extracts were immunoprecipitated with T7-tag antibody, recognizing the recombinant protein, run in SDS-gel, transferred to PVDF membrane, and pol-prim-associated proteins were detected by the indicated antibodies as western blotting primary antibody. All the immunoprecipitation steps were the same as described in section 2.2.4 and western blotting steps were the same as mentioned in section 2.2.10.

2.2.5 Transfection

Transient transfection using PolyFect transfection reagent

HEp 2 and HEK 293 cells were transiently transfected at about 75% confluency using PolyFect Transfection Reagent (Qiagen). Cells were seeded for 24 hrs. before transfection. The following protocol as per the recommendation of manufacturer was followed.

Table 2.

Culture format	No. of cells to seed	Vol. of medium (µl)	DNA (µg)	Final vol. of diluted DNA (µl)	Vol. of PolyFect Reagent (µl)	Vol. of medium to add to cells (µl)	Vol. of medium to add to complexes (µl)
6-well plate	4×10^5	3000	2	100	22	1500	600
60 mm dish	8×10^5	5000	4	150	45	3000	1000
100 mm dish	1.6×10^5	8000	8	300	90	7000	1000

2.2.6 Labeling of oligonucleotides

Radio-labeling reaction was performed according to standard protocol (Sambrook et al., 1989). 10 ng/ul oligonucleotide was mixed with 5 µl of 10x PNK buffer, 7.000 Ci/mmol of $^{32}P\gamma ATP$ and 10 units/µl of polynucleotide kinase (PNK). Adjusted the total volume to 50 µl with water and incubated for one hr. at 37°C. ProbeQuant™ G-50 micro columns were used for the removal of unincorporated nucleotides from the labeled oligonucleotids. First the resin was resuspended in column by vortexing. Then the cap was loosened, the tube was one-fourth turned and the bottom closure was snapped off. Prior to use the column, it was centrifuged for 1 min. at 3000 rpm. Then the column was placed in a new 1.5 ml tube and 50 µl of the sample were slowly loaded on to the resin. Finally the column was centrifuged at 3000 rpm for 2 min. and purified sample was collected.

PNK Buffer
70 mM Tris/HCL (pH 7.6)
10 mM $MgCl_2$
5 mM dithiotreitol

2.2.7 Electrophoretic mobility shift assay

When proteins bind to DNA molecules, the resulting protein-DNA complex reveals a large increase in overall molecular mass compared to the free DNA. In practice a DNA associated with protein is separated from the free DNA by gel electrophoresis. The procedure is referred as gel retardation or electrophoretic moblity shift assay (EMSA).

Mixed different concentrations of proteins with 1 ng oligonucleotides, binding buffer (final concentration: 10 mM Tris/HCl pH 7.5 and 5 mM EDTA, 50 mM NaCl and 1 mM BSA). After mixing incubated at 25°C for one hr. then added 5µl loading dye and run in 6% native polyacrylamide gel at 70 V in 0.5 X TBE buffer (0.045 M tris-borate, 0.001 M EDTA) overnight until the bromophenol blue is 2.5 cm above from the bottom of the gel. Then the gel was dried at 80°C for 2 hrs. and exposed to film or phosphorimager (Fujifilm Europe, FLA-5100) for autoradiography.

2.2.8 SDS polyacrylamide gel electrophoresis

Denaturing polyacrylamide gel electrophoresis (PAGE) was used to analyze the molecular mass of proteins and protein complex composition. SDS polyacrylamide gel electrophoresis (size of the gel was 80mm x 70mm x 10mm) was carried out with Biometra minigel apparatus (Göttingen). Buffers were used according to (Laemmli, 1970). The stacking gel contained 0.1% (w/v) SDS, 4% (w/v) bisacrylamide, 130 mM Tris/HCl pH 6.8, 0.09% (w/v) APS and 0.3% (v/v) TEMED. The separating gel contained 0.1% (w/v) SDS, 10% (w/v) bisacrylamide, 380 mM Tris/HCl pH 8.8, 0.05% (w/v) APS and 0.12% (v/v) TEMED.

4 x SDS loading buffer

40 mM Tris/HCl, pH 6.75
4% SDS
10% 2 β-Mercaptoethanol
40% Glycerin
0.002% Bromophenol blue

1x Laemmli running buffer

25 mM Tris
193 mM Glycin
0.1% SDS

2.2.9 Coomassie^{R} Brilliant Blue staining of protein

Coomassie^{R} Brilliant Blue dye was used for staining of proteins in SDS gel. Gel was incubated in Coomassie blue solution for 30 min. with slight agitation, followed by incubation in destaining solution (40% methanol, 10% acetic acid) until protein bands were visible on a colorless background. Gel was then incubated in gel drying solution (20% ethanol, 10% glycerin in water) for 30 min., and put on Whatman 3mm paper, covered with cellophane, and dried by a gel dryer at 80ºC for 4 hrs.

2.2.10 Western blotting

Polypeptides, which were separated by SDS-PAGE were transferred to solid support (PVDF membrane). The proteins on the membrane were then probed with specific antibodies.

2.2.10.1 Proteins transfer and immunoreaction

Prior to proteins transfer PVDF membrane was pre-wet in 100% methanol for 15 sec. Membrane was equilibrated for at least 5 min in transfer buffer. The proteins were transferred to membrane with 150 mA for one hr. Then the membrane was incubated in blocking buffer for one hr. to block the non-specific binding sites. The membrane was washed twice with TBST for 10 min. and incubated in primary antibody, which was optimally diluted in antibody dilution buffer for 1 hr. at room temperature. Membrane was then washed 3 times with TBST, each time for 15 min. After the washing steps, the membrane was incubated for 1 hr. with

optimally diluted secondary antibody at room temperature for 1 hr. The membrane was washed 3 times with TBST and developed either by ECL or colorimetric method depending on the type of secondary antibody.

2.2.10.2 Detection of proteins in western blotting

2.2.10.2.1 Luminescence-based detection ECL

The blots were developed using the ECL™ chemiluminescent detection reagents (GE Health previously Amersham Bio-sciences). The detection solution was prepared by mixing equal volumes of solution 1 with solution 2 to yield sufficient volume to cover the membrane (~0.123ml/cm^2 of membrane, 1 ml total). Immediately after mixing, the solution was poured directly onto the protein containing side of the blot surface and incubated for 1 min. at room temperature. The blot was then removed from the detection solution and excess solution removed by touching the edge of the blot against a piece of tissue paper. The blot was enclosed in plastic wrap and exposed to film (Kodak® BioMax® chemiluminescence film) up to 30 seconds.

2.2.10.2.2 Colorimetric detection

The blot membrane was incubated in 20 ml AP-buffer containing 66ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 132 ml nitro blue tetrazolium (NBT), protected from light with aluminium foil. The substrate BCIP/NBT was converted into a dense blue compound by alkaline phosphatase. When the desired signal strength was obtained, the staining solution was washed out with PBS twice to stop the reaction.

2.2.10.2.3 Stripping and reprobing

The membrane was submerged in stripping buffer and incubated at 50°C for 30 min. with occasional agitation. Next the membrane was washed twice with TBST. The membrane was then blocked by immersing in 5% blocking reagent for 1 hr.

at room temperature. Immunodetection was performed as described above (section 2.2.10).

TBST buffer 10x
250 mM Tris/HCl, pH 7.4
1.37 M NaCl
27 mM KCl
0.5% (v/v) Tween-20

AP buffer
100 mM NaCl
5 mM $MgCl_2$
100 mM Tris/HCl, pH 9.0

Transfer buffer
39 mM glycine
48 mM Tris base
0.037% SDS
20% methanol

TBS
50 mM Tris/HCl, pH 7.5
150 mM NaCl

Blocking buffer
5% fat-free milk powder (g/ml) in TBST

Antibody dilution buffer
5% FCS in TBST

BCIP stock solution
0.5 g of BCIP disodium salt in 10 ml of 100% dimethylformamide

NBT stock solution
0.5 g NBT in 10 ml of 70% dimethylformamide

Stripping buffer
100 mM 2-mercaptoethanol
2% sodium dodecyl sulphate (SDS)
62.5 mM Tris/HCl, pH 6.7

2.2.11 Purification of recombinant human primase

Primase (p48/p58) and C-terminus of p58 (CTp58) were expressed in *E. coli* BL(DE3) using the vectors, pET-His Hp58-Hp48 and pET11p48-Hisp58CT, respectively. *E. coli* cells BL21(DE3) containing the expression vector were grown in LB broth at 37°C (Sambrook et al., 1989) to an optical density of ~0.5 at 600 nm. After lowering the temperature to 23°C, the expression of recombinant proteins was induced by the addition of 1mM isopropyl-1-thio-B-D-galactopyranoside (IPTG). The cells were harvested 3.5 hrs. after induction, collected at 15,000 x g, and washed twice with phosphate buffered saline. Alternatively, proteins were also expressed in the presence of 2,2'-dipyridyl (200 mM) to complex iron. *E. coli* cells were quick frozen on dry ice and stored at -20°C until purification. Frozen cells were quickly thawed and homogenized in lysis buffer (50mM Tris/HCl, pH 8.0, 150 mM NaCl, 3 mM 2-mercaptoethanol, 0.05 mM Leupeptin, and 0.01 mg/ml Trasylol) by sonication. After sonication the homogenate was adjusted to 1% Triton X-100. The suspension was mixed well and incubated on ice for 30 min. with mixing intermittently. Following the 30 min. incubation on ice ~60 units of DNase (Sigma) was added to the suspension and incubated for a further 30 min. on ice with mixing frequently. Following incubation the suspension was centrifuged (15,000 rpm for 15 min. at 4°C) to remove DNA and insoluble proteins. The supernatant was applied to metal chelate chromatography using Sigma Ni Resin. After binding of the His-tagged protein to the affinity resin, the resin was washed extensively with buffer 1 (20 mM Tris/HCl, pH 8, 250 mM NaCl, 3.5 mM 2-mercaptoethanol, 1% Triton X-100; about 50 column volumes). In the next step, the resin was washed with 5-column volumes of each buffer 2 (100 mM $(NH_4)_2SO_4$, pH 8.2) plus 10mM imidazole and buffer 2 plus 20mM imadazole. Proteins were eluted with 100 mM imidazole in buffer 2.

2.2.12 Primase assay

The reaction mixtures for the coupled DNA polymerase I-primase assay contained 20 mM Tris acetate, pH 7.3, 10 mM magnesium acetate, 0.1 mM poly(dT), 0.05 mM [α-^{32}P] dATP (30 cpm/pmol), 1 mM ATP, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 25 units/ml DNA polymerase I (large fragment). 0.2-2 units of primase were incubated with 20 μl of the assay mixture for 5 and 10 min. The incorporated radioactivity was measured by pipetting aliquots on GF35 filter discs (Schleicher & Schull) and immersing the discs in ice-cold 10% trichloroacetic acid for 10 min. Discs were then washed four times with 1 M HCl and two times with ethanol, dried, and counted in a toluene-based scintillation mixture using a scintillation counter.

2.2.13 DNA polymerase assay

DNA polymerase activity was measured in a 50 μL reaction containing 20 mM Tris-acetate, pH 7.3, 75 mM potassium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM each of dGTP, dCTP, dTTP and [α-^{32}P] dATP (10 cpm / pmol), and 100 μg / mL BSA, 0.2 mg/mL activated DNA. The reaction was started by adding 1 unit of DNA polymerase. Incubation was carried out at 37°C for 10 min. The incorporated radioactivity was measured by pipetting 50 μL on GF35 filter discs (Schleicher & Schull) and immersing the discs in ice-cold 10% trichloroacetic acid for 10 min. Discs were then washed four times with 1 M HCl and two times with ethanol, dried, and counted in a toluene-based scintillation mixture using a scintillation counter.

3 Results

DNA polymerase α and primase are essential proteins of the cellular DNA replication machinery. They are important components of the initiation complex and play a central role in leading and lagging strand synthesis at the DNA replication fork. Primase synthesizes oligoribonucleotides, called RNA primers for both leading and lagging strands (Foiani et al., 1997; Roth, 1987; Wang, 1991). The enzyme consists of two subunits with molecular masses of 58 and 48 kDa, and is associated with the 180 kDa DNA polymerase subunit and a 70 kDa subunit in eukaryotic cells (Conaway and Lehman, 1982; Kaguni et al., 1983). To date, the precise function of the p58 subunit is not clear. Previous studies have revealed that p58 stabilizes primase activity of p48, increases the rate of primer synthesis and can bind ssDNA as well as the partially duplex RNA-DNA product formed after primer synthesis (Hubscher et al., 2002; Kirk et al., 1997; Nasheuer and Grosse, 1988; Sheaff and Kuchta, 1993). In the current work, attempts were made to analyze the binding affinity of primase for different DNA substrates and the recognition of sequence in p58, responsible for binding to different substrates.

3.1 Expression and purification of recombinant human primase

The cDNAs coding for the p58 subunit, p48 subunit and the deletion mutant of p58, CTp58 (Schlott and Nasheuer, unpublished results summarized in Figure 3.1A) were fused to six histidine residues at their N terminus and expressed using bacterial expression vectors. The proteins were subsequently purified by Ni columns. The His-tagged p58 subunit was co-expressed with the p48 subunit (without a tag) using a promoter producing a polycistronic mRNA (Schneider et al., 1998). Co-expression of both primase subunits allowed the purification of a heterodimer in high yields (Figure 3.1B). Average yield was about 3 mg /l of bacterial culture. The C-terminus of p58 was produced in even higher quantities resulting in about 5 mg/l cell culture. The purified proteins were analyzed

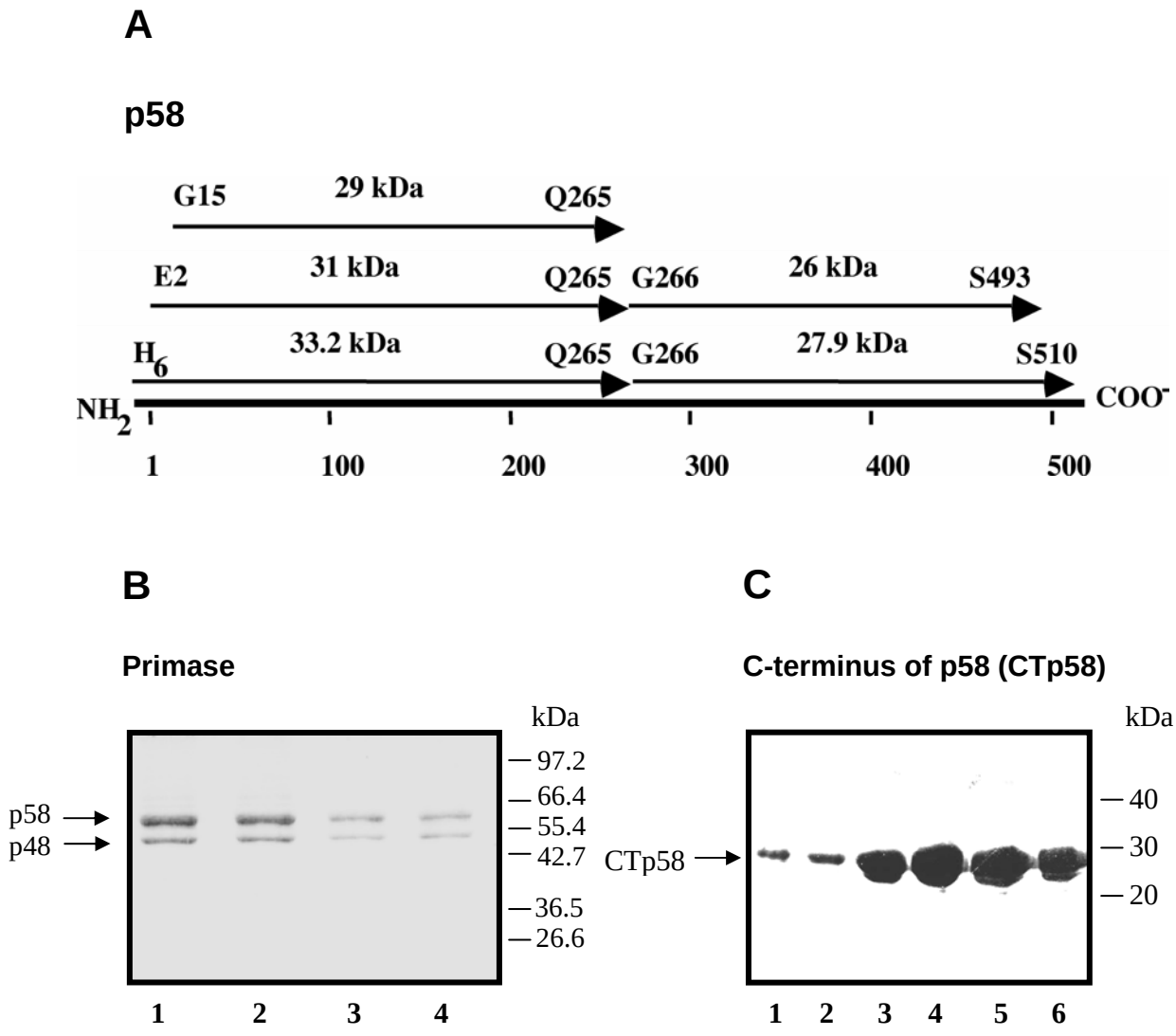

Figure 3.1: (A) Schematic map of proteolytic peptides generated upon primase treatment with trypsin. Proteolytical stable domains of human primase were determined by partial tryptic digest of 10 µg of primase (Schlott and Nasheuer, unpublished data). The proteolysis products were then analyzed by SDS gel electrophoresis and peptide sequencing as described earlier and are shown in panel A (Pestryakov et al., 2003). **(B and C) Purification of recombinant proteins.** Primase p48/p58 and C-terminus of p58 (CTp58) with p58 and CTp58 being fused to a His6 tag and p48 being untagged were expressed in *E. coli* BL21(DE3). Proteins were purified using metal chelate chromatography (Sigma Ni resin) and eluted fractions were subjected to SDS gel electrophoresis. (A: H_6 is 6 Histidine tag, B: lanes 1 to 4, primase dimer and protein marker is from New England BioLabs, broad range 2-212 kDa and C: lanes 1 to 6, CTp58 and marker is from Gibco/BRL, 10 kDa ladder).

by SDS-gel electrophoresis as shown in Figure 3.1B and 3.1C. These results show that these proteins were purified to near homogeneity.

3.2 Primase binding to M13-ssDNA

DNA polymerase α-primase first binds a DNA template before synthesizing RNA primers (Frick and Richardson, 1999; Thompson et al., 1995). To further characterize primase bindings, we performed gel mobility shift assays using M13-ssDNA as a substrate. Increasing amounts of purified primase were incubated with a fixed amount of M13-ssDNA. Protein-DNA complexes were then resolved on agarose gels and DNA was visualized by ethidium bromide staining. As shown in Figure 3.2A, incubation of the M13-ssDNA with primase dimer caused a shift in its gel mobility. The shift in electrophoretic mobility increased in a concentration-dependent manner. Similarly, primase subunit p48 also showed binding with M13-ssDNA (Figure 3.2B). As primase subunit (p58) is known to bind ssDNA (Arezi et al., 1999), we carried out EMSA to determine the region of p58 which binds to M13-ssDNA. As shown in Figure 3.2C, the C-terminus of p58 (CTp58, starting with G266 and ending with residue S510) binds to M13-ssDNA, and as with the whole protein (p48/p58), this binding is dose-dependent. The apparent dissociation constants of primase dimer, p48 and CTp58 were 0.5 nM, 2 nM and 2 nM, respectively. The dissociation constant of primase-M13-ssDNA was in a similar range as that of RPA (0.2 nM, Figure 3.2). Moreover, primase bound the natural ssDNA of øX174 with a similar affinity as M13-ssDNA.

3.3 Primase and its subunits bind to oligonucleotides

To characterize the substrate, which is bound by primase, in an optimal manner, we used oligo-pyrimidine (oligo Py) and oligo-purine (oligo Pu, Table 1), since in enzyme assays primase preferentially utilizes oligo-pyrimidines (Frick and Richardson, 1999). However, primase did not bind any of the two oligonucleotides. Then both oligonucleotides, oligo Pu and oligo Py, were annealed to produce double-stranded oligonucleotides (ds pupy oligo 80) to analyze whether primase interacts with double-stranded oligonucleotide.

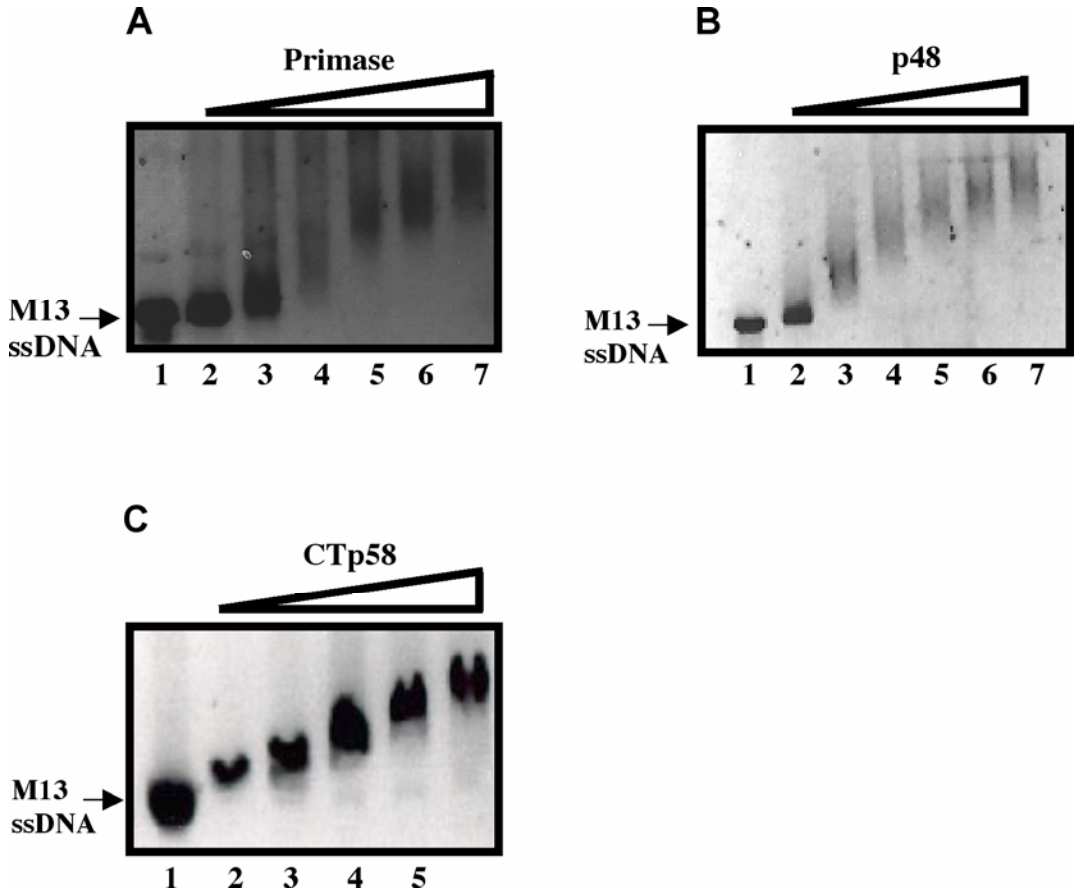

Figure 3.2: Binding of primase dimer (p48/p58) and its components (p48, CTp58) to M13-ssDNA. Electrophoretic mobility shift assay (EMSA) was carried out to analyze primase binding with M13-ssDNA. **(A)** Primase (p48/p58) binds to M13-ssDNA. 0.14 μg M13-ssDNA were incubated with no protein lane 1 or increasing amounts (0.25, 0.5, 0.75, 1, 2 and 3 μg) of purified primase (lanes 2 to 7, respectively). **(B)** Primase subunit p48 binds to M13-ssDNA. 0.14 μg M13-ssDNA were incubated with no protein lane 1 or increasing amounts (1, 2, 3, 4, 5 and 6 μg) of purified primase subunit p48 (lanes 2 to 7, respectively). **(C)** C-terminus of p58 (CTp58) binds to M13-ssDNA. 0.14 μg M13-ssDNA were incubated with no protein lane 1 or increasing amounts (1, 2, 3, 4 and 5 μg) of purified CTp58 (lanes 2 to 6, respectively). The protein-DNA complexes were separated by 0.8% agarose gel electrophoresis. The DNA was then stained with Ethidium bromide.

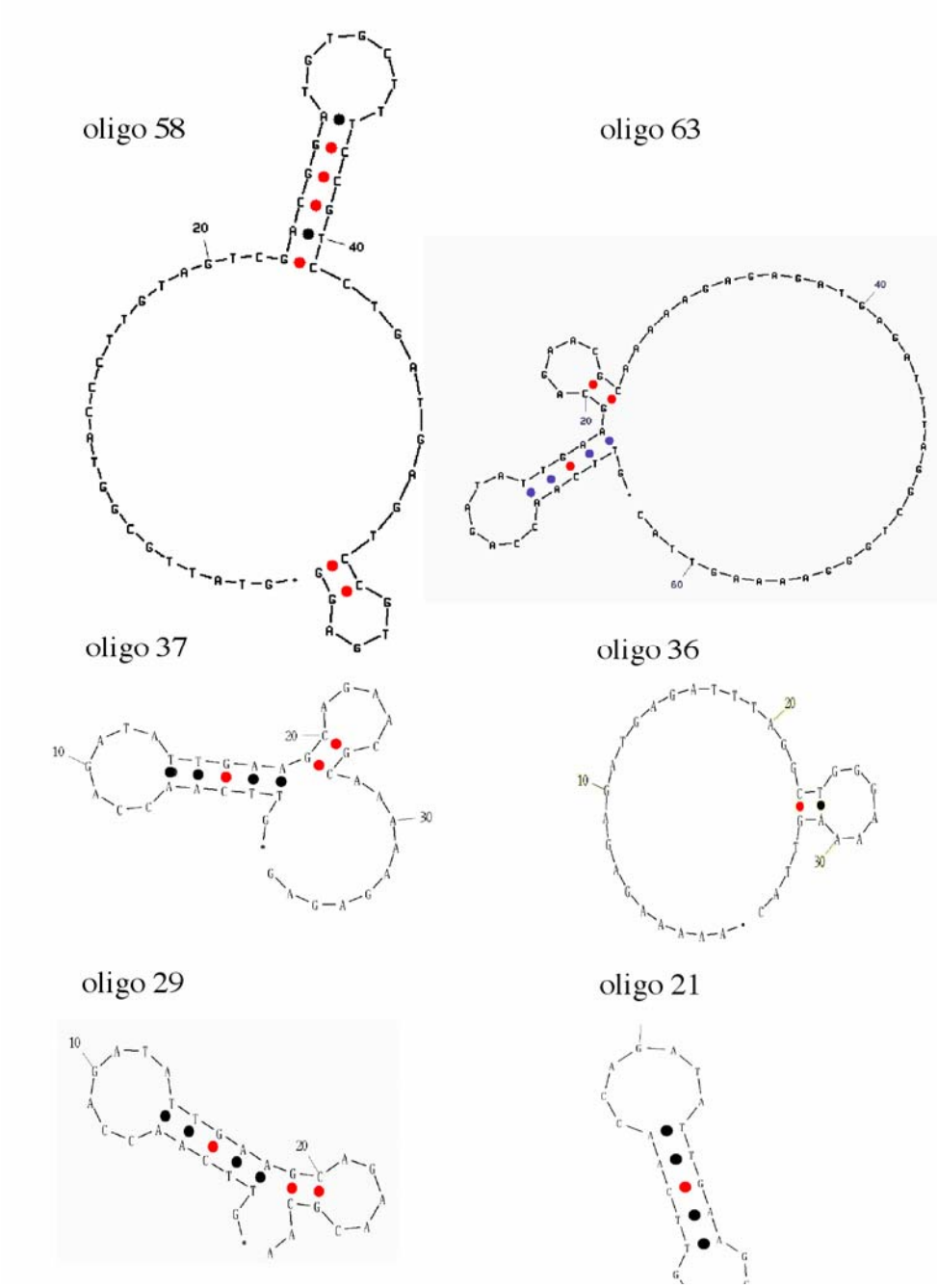

Figure 3.3: Predicted structures of oligonucleotides. (http://207.32.43.70/biotools/oligocalc/ oligocalc.asp). Oligonucleotide oligo 58 was designed with 2 hairpins. Oligonucleotide oligo 63 is a sequence of the bacteriophage øX174 and the computer program of the above website predicts that it contains 2 hairpin structures. The oligonucleotides oligo 37, oligo 36, oligo 29 and oligo 21 were derived from oligo 63. Oligonucleotides oligo 29 and oligo 37 make the main secondary structures (position 2 to 27) like oligonucleotide oligo 63, while oligonucleotide oligo 36 also contains a minor secondary structure which has very low melting temp. of 11.7°C. Oligonucleotide oligo 21 forms one hairpin structure (position 2 to 18). Properties of different oligonucleotides are as:

Oligonucleotides	oligo 58	oligo 63	oligo 37	oligo 36	oligo 29	oligo 21
dG (kcal.mole^{-1})	-5.88	-1.64	-1.64	0.93	-1.64	-0.91
dH (kcal.mole^{-1})	-70.6	-54.4	-54.4	-19.2	-54.4	-40.8
dS (cal.K^{-1}mole^{-1})	-217.0	-177.1	-177.1	-67.4	-177.1	-133.8
T_M (°C)	52.2	34.0	34.0	11.7	34.0	31.7

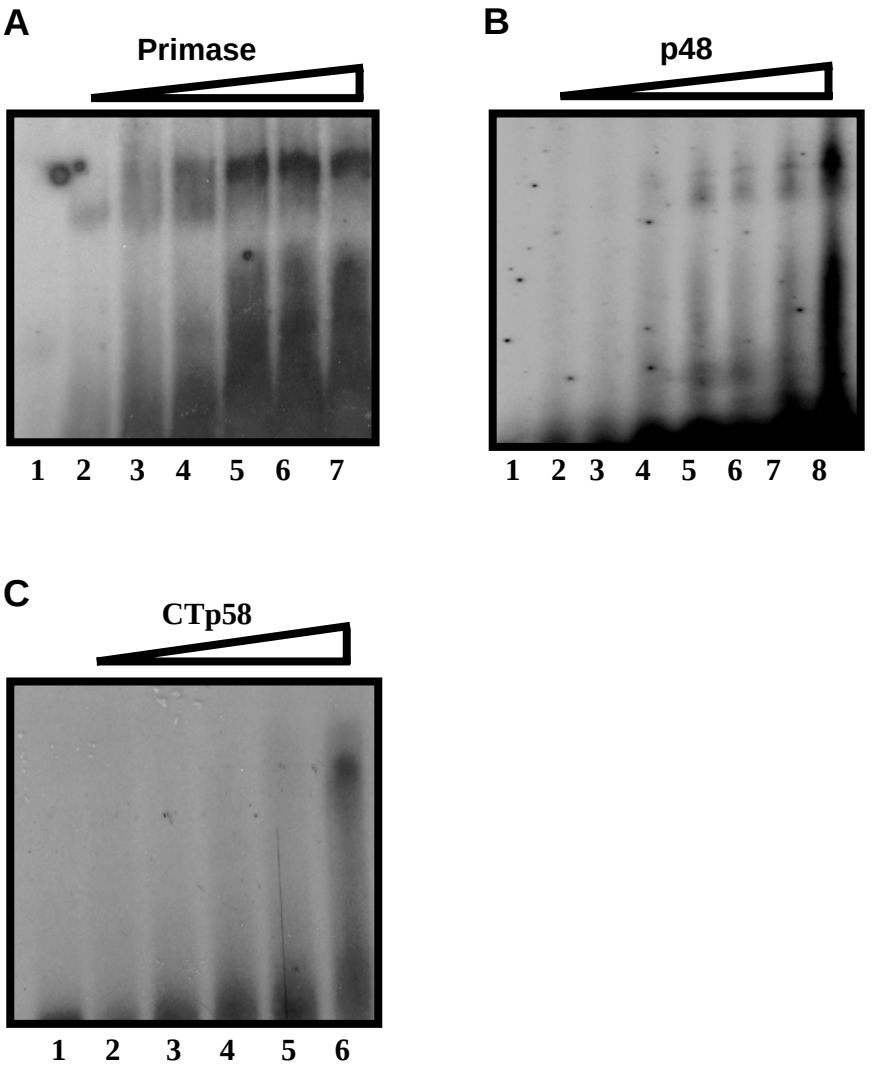

Figure 3.4: Primase (p48/p58) and its components (p48, CTp58) bind to oligonucleotides. (A) Primase binds to oligonucleotide oligo 58. Increasing amounts (1, 2, 3, 4, 5 and 6 μg) of purified primase p48/p58 (lanes 2 to 7, respectively) were incubated with radioactively labeled oligonucleotide oligo 58. **(B)** Small subunit p48 of primase binds to oligonucleotide oligo 58. Increasing amounts (1, 2, 3, 4, 5, 6 and 7μg) of purified p48 (lanes 2 to 8, respectively) were incubated with radioactively labeled oligonucleotide oligo 58. In case of p48 binding with oligo 58, a higher amount of purified protein (7μg) was used. **(C)** C-terminus of p58 (CTp58) binds to oligonucleotide oligo 58. Increasing amounts (1, 2, 3, 4 and 5 μg) of purified CTp58 (lanes 2 to 6, respectively) were incubated with radioactively labeled oligo 58. The protein-DNA complexes were separated by non-denaturing gel electrophoresis (6% acrylamide). Bound and free DNA was determined by autoradiography. Only the shifted protein-DNA complexes are presented here since the free DNA was over-exposed. For comparison, the gel shift of oligonucleotide oligo 58 was also analyzed by non-denaturing gel electrophoresis and autoradiography in the presence of only assay buffer (lane 1) in all three gels.

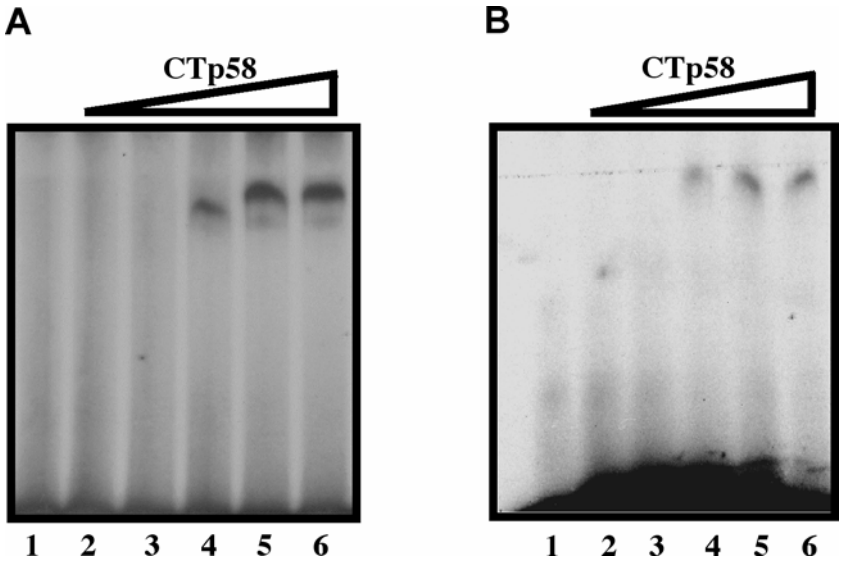

Figure 3.5: C-terminus of p58 (CTp58) binds to oligonucleotides, oligo 63 and double stranded pupy oligo 80. (**A)** C-terminus of p58 (CTp58) binds to oligonucleotide oligo 63. Increasing amounts (1, 2, 3, 4 and 5 μg) of purified CTp58 (lanes 2-6, respectively) were incubated with radioactively labeled oligonucleotide oligo 63. (**B)** C-terminus of p58 (CTp58) binds to ds pupy oligonucleotide, oligo 80. Increasing amounts (1, 2, 3, 4 and 5 μg) of purified CTp58 (lanes 2-6, respectively) were incubated with radioactively labeled double stranded pupy oligonucleotide oligo 80. The protein-DNA complexes were separated by non-denaturing gel electrophoresis (6% acrylamide). Bound and free DNA was determined by autoradiography. Only the shifted protein-DNA complexes are presented here since the free DNA was over-exposed. For comparison, the gel shift of oligonucleotide oligo 63, and oligonucleotide oligo 80, were also analyzed by non-denaturing gel electrophoresis and autoradiography in the presence of only assay buffer (lane 1). In case of CTp58 binding to double stranded oligonucleotide oligo 80, very faint bands appeared only after exposing for a very long time (96 hrs.), whereas in case of gel A, the exposure time was only 16 hrs. The single-strand oligonucleotides (80 nucleotides long) consisting of oligo-pyrimidine strand (oligo Py) or of oligo-purine (oligo Pu) were not bound by CTp58 even in the highest concentration and long exposure times (data not shown).

This analysis showed that CTp58 recognized ds pupy oligonucleotide oligo 80 with low affinity and very faint bands appeared after a very long exposure (96 hrs.) as shown in Figure 3.5B. The binding of primase (p48/p58) and its subunits p48, p58 and 'C' terminus of p58 "CTp58" with poly(dT) were also checked, but no significant binding was observed (data not shown). In contrast to this result, M13-ssDNA is efficiently recognized (Figure 3.2). However, although the M13 phage genome contains single-stranded DNA, it is not a simple linear single strand of DNA, as it contains various structures including hairpins, and stem loops. Therefore, oligonucleotides were designed to determine the structure required for primase binding. These oligonucleotides of different lengths and structures were radioactively labeled and then used as probe for primase (p48/p58) binding in gel shift assays. We choosed an oligonucleotide oligo 58 that can form hairpin structures (Figure 3.3) as predicted by the biotools program (http://207.32.43.70/biotools/oligocalc/oligocalc.asp) and determined by NMR (M. Görlach, personal communication). A fixed amount of radioactively labeled oligonucleotide oligo 58 (see table 1 for sequence and Figure 3.3 for predicted structure) was incubated with increasing amount of primase (p48/p58) or primase subunit (p48). The protein-DNA complexes were separated by non-denaturing gel and free DNA and protein-DNA complexes were determined by autoradiography. Both primase (p48/p58) and primase subunit (p48) showed binding to oligonucleotide oligo 58 and this binding was increased in a concentration-dependent manner (Figure 3.4A and B). Similarly the binding of CTp58 with oligonucleotide oligo 58 was also checked and Figure 3.4C clearly shows that the CTp58 also binds to the oligonucleotide oligo 58. Protein concentrations are same in lane 7 of both gels Figure 3.4A and Figure 3.4B, but binding seems to be quite reduced in case of p48 subunit. This interaction is further reduced in the case of CTp58 binding to oligonucleotide oligo 58 (Figure 3.4C).

An additional oligonucleotide oligo 63 was designed using øX174ss-DNA sequence (From position 5231 to position 5293), since øX174ss-DNA was as efficiently bound by primase as M13-ssDNA (data not shown). As presented in Figure 3.5A CTp58 binds oligo 63 with high affinity. This DNA binding of primase can be sequence-specific or structure-specific. To discriminate between these alternatives, different oligonucleotides were designed from oligonucleotide oligo 63, for further binding studies.

3.4 CTp58 binds to oligonucleotides

Oligonucleotides (Table 1) were designed from the sequence of oligonucleotide oligo 63 (derived from øX174 DNA sequence), to analyze which specific sequences or structures are more efficiently bound. These oligonucleotides form different hairpin type structures as shown in Figure 3.3 (http://207.32.43.70/biotools/oligocalc/oligocalc.asp). Binding assays showed (Figure 3.6) that CTp58 binds more efficiently to oligonucleotides oligo 29 and oligo 37. However, complex formation of these shorter oligonucleotides with primase was only observed after glutaraldehyde cross-linking, suggesting that the association of primase with this DNA is less stable than the binding to long oligonucleotides such as oligonucleotides oligo 58 and oligo 63 and that the high affinity DNA binding activity of primase may need secondary structure and flanking sequences.

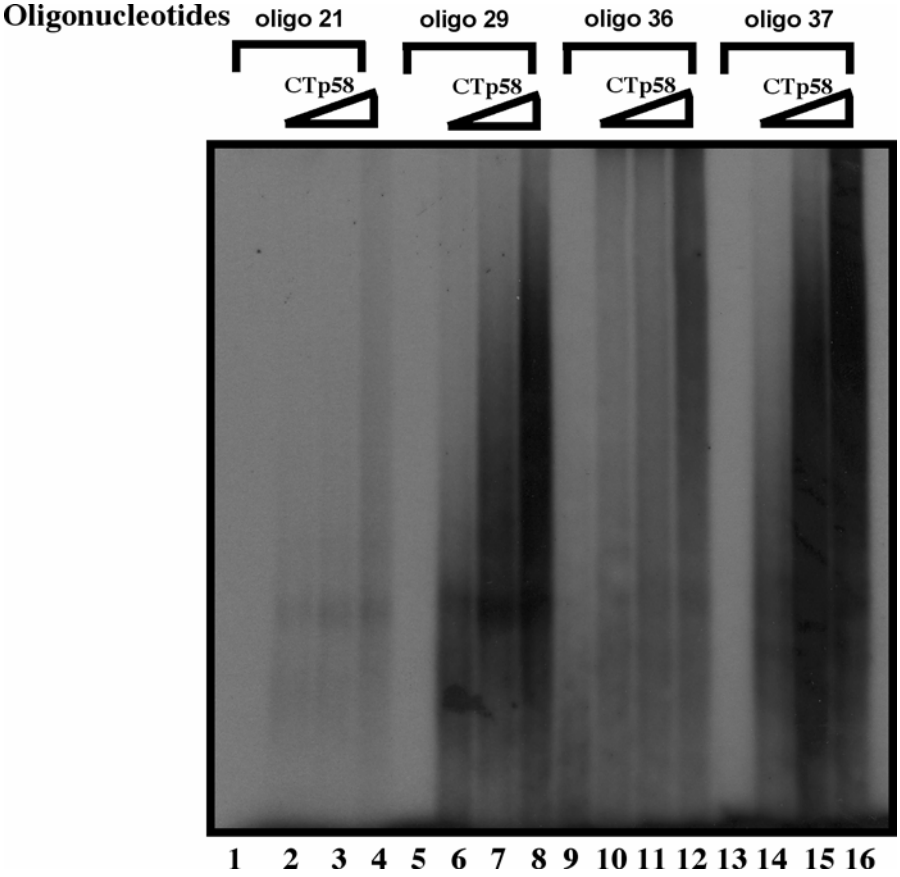

Figure 3.6: Binding affinity of C-terminus of p58 (CTp58) to various oligonucleotides. CTp58 binds differently to structured oligonucleotides (see Figure 3.3). Increasing amounts (1, 2 and 4 μg) of purified CTp58 (lanes 2 to 4, 6 to 8, 10 to 12, and 14 to 16, respectively) were incubated with radioactively labeled oligonucleotides (oligo 21, oligo 29, oligo 36 and oligo 37). Glutaraldehyde was also added in a final concentration of 0.1%. The protein-DNA complexes were separated by non-denaturing gel electrophoresis (6% acrylamide). Bound and free DNA was determined by autoradiography. Only the shifted protein-DNA complexes are presented here since the free DNA was over-exposed. For comparison, the gel shifts of different oligonucleotides were also analyzed by non-denaturing gel electrophoresis and autoradiography in the presence of only assay buffer (lanes 1, 5, 9 and 13).

3.5 Inhibition of primase activity in the presence of oligonucleotides

Primase activity using poly(dT) as template was carried out in the presence of oligonucleotides (oligo 21, oligo 36, oligo 37, and oligo 58). The Klenow DNA polymerase elongation assay was used as previously described (Nasheuer and Grosse, 1988). Inhibition in primase activity was observed in the presence of oligonucleotides and this inhibition was concentration-dependent (Figure 3.7A). Inhibition in primase activity correlates well with the ability of primase to bind to a primer (compare Figures 3.4, 3.6 and 3.7). The primer, which was preferentially recognized by primase, also most strongly inhibits primase activity. In addition, this inhibition pattern is consistent over the whole concentration range of the oligonucleotides. Primase activity in the absence of oligonucleotides was measured and set as 100%, other activities were taken with reference to this activity. The addition of 2 μM competitor oligonucleotides significantly reduces primase activity. The most effective oligonucleotide was oligo 58 and the least effective oligonucleotide was oligo 21 (5% and 45%, respectively remaining enzyme activity). This corresponds to the binding ability of these two oligonucleotides with primase. The primase activities in the presence of 2 μM of oligo 36 and 37 were 25% and 8%, respectively, in comparison to the enzyme activity in the absence of oligonucleotides. With increasing concentration of competitors, primase activity also decreases. At 4 μM oligonucleotide concentration, primase activity was about 30% with oligonucleotide oligo 21 and 15% with oligonucleotide oligo 36 whereas oligonucleotides oligo 37 and oligo 58 reduced primase activity to a higher degree (6% and 4%, respectively). The functional primase assay show that the binding result in the EMSA is consistent with the inhibitory effect of the tested oligonucleotides. DNA polymerase activity was also measured in the presence of these oligonucleotides (oligo 21, oligo 36, oligo 37 and oligo 58). None of the oligonucleotide inhibited activity of DNA polymerase I large fragment (Klenow enzyme), which suggests that the inhibition by oligonucleotides is primase-specific (Figure 3.7B).

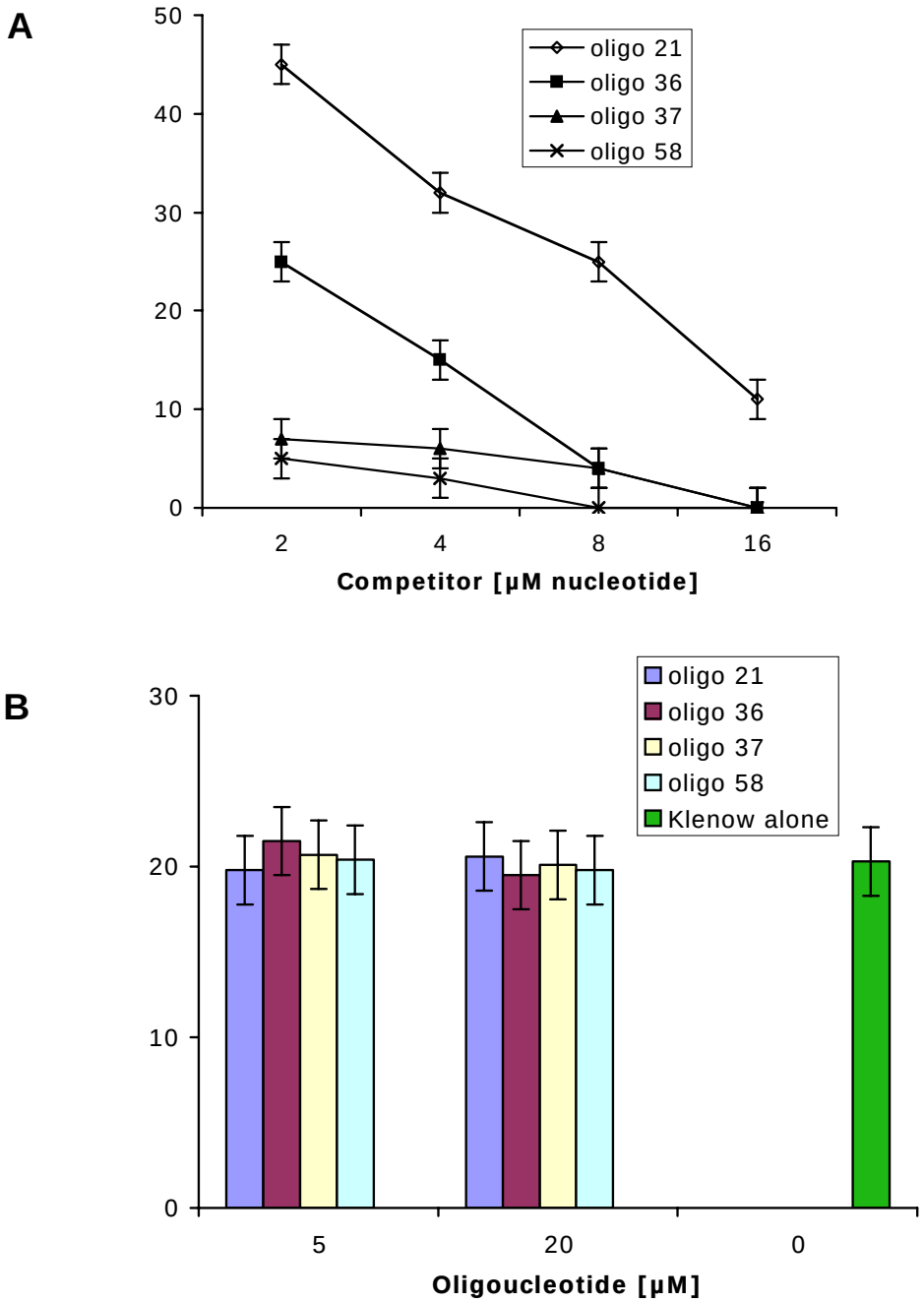

Figure 3.7: Primase and DNA Polymerase assays in the presence of oligonucletides. (A) Comparison of inhibition of primase activity by oligonucleotides. Primase activity using poly(dT) as template was carried out in the presence of oligonucleotides (oligo 21,oligo 36, oligo 37, oligo 58) at increasing concentrations. The DNA polymerase elongation was used and carried out in the presence of [α-^{32}P] dATP according to (Nasheuer and Grosse, 1988) as described in Materials and Methods section 2.2.12. The mean and standard deviation of three experiments are presented. **(B) DNA Polymerase assay in the presence of oligonucleotides.** DNA polymerase activity of bacterial DNA polymerase I (large fragment) was measured according to (Nasheuer and Grosse, 1987) as described in Materials and Methods section 2.2.13. The mean and standard deviation of three experiments are presented.

3.6 Replication protein A (RPA) and primase co-operate in their binding to DNA

The interactions of the heterotrimeric RPA complex with ssDNA have been thoroughly investigated previously (Iftode et al., 1999; Wold, 1997). In present study experiments were designed to study whether RPA influences the binding of primase with DNA and vice versa. Therefore, first the binding of RPA with M13-ssDNA was analyzed. This analysis confirmed that RPA efficiently binds to M13-ssDNA as shown in Figure 3.8A and the dissociation constant was 0.2 nM. Lane 1 was loaded with M13-ssDNA (without protein) while in lanes 2-6 increasing amounts of RPA were incubated with fixed amount of M13-ssDNA. To analyze whether primase stimulates binding of RPA a constant amount of primase, which does not show a shift in the mobility of M13-ssDNA (lane 8), was added to the RPA assay. In addition of RPA, primase was also added in lanes 9-13. Interestingly, with the addition of a fixed amount of primase (0.25μg), a substantial increase in the binding of RPA to M13-ssDNA was detected and a protein-M13ssDNA complex could be determined with concentration of RPA and primase that reproducibly did not shift DNA alone. Moreover, even at higher concentrations of RPA the mobility of the protein DNA complex was decreased in comparison with the shift of the same protein concentrations in the absence of primase. This finding suggests that even low amounts of primase co-operate with RPA to interact with M13-ssDNA.

To further investigate the effect of RPA on primase and DNA binding, a fixed amount of RPA was added in primase-M13-ssDNA binding assay (Figure 3.8B). Lanes 1 and 4 contain only M13-ssDNA (without any protein), while lanes 2-3 and 6-7 contain increasing amounts of primase. The difference between lanes 2-3 and 6-7 is that in lanes 6-7 a fixed amount of RPA was added, which significantly stimulated primase binding to M13-ssDNA as determined by the

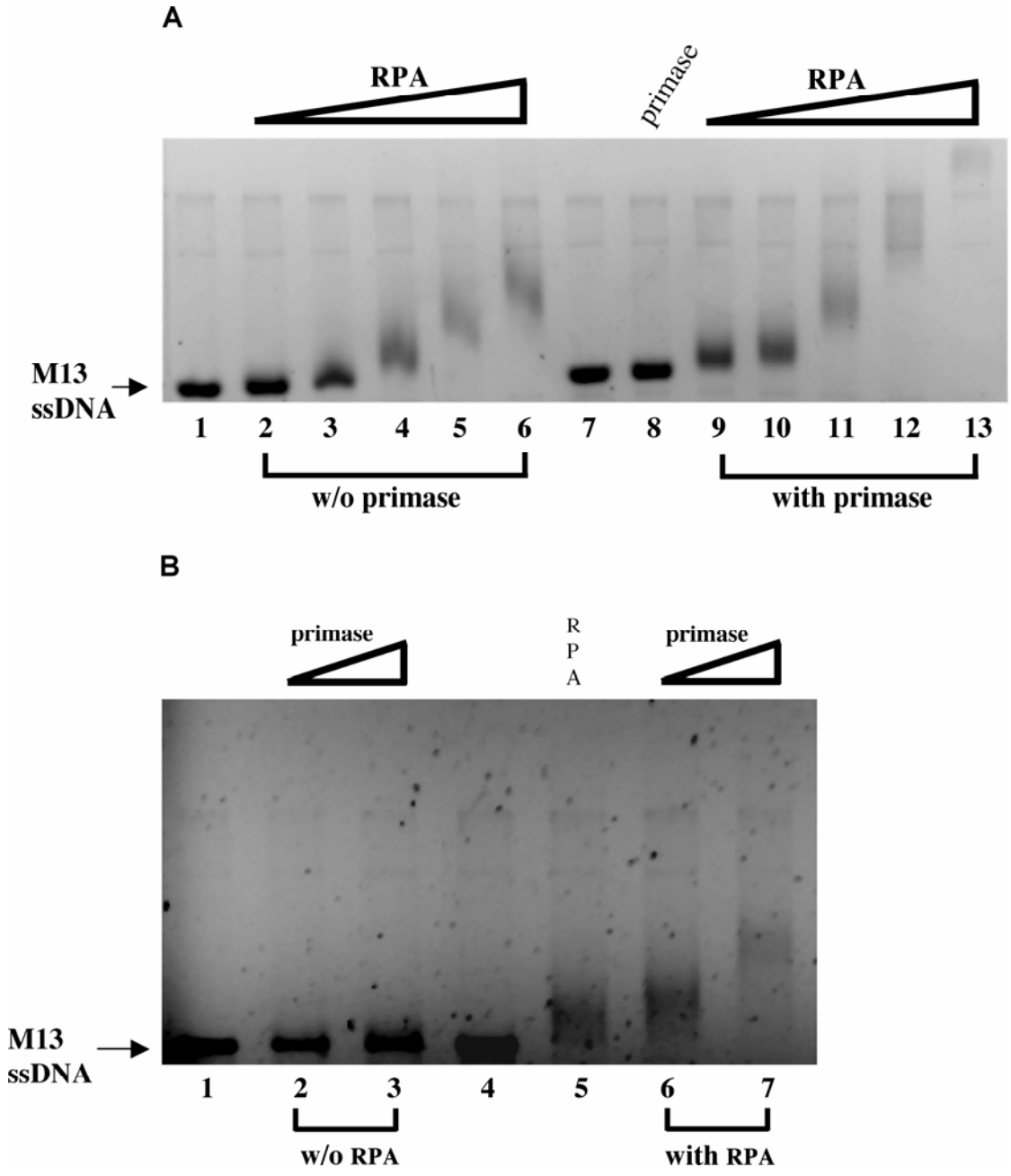

Figure 3.8: Primase and RPA co-operate in their binding to M13-ssDNA. (A) Primase stimulates RPA binding to M13-ssDNA. 0.14 μg M13-ssDNA were incubated with no proteins (lanes 1 and 7) or increasing amounts (0.25, 0.5, 1, 1.5 and 2 μg) of RPA (lanes 2 to 6 and 9 to 13 respectively). In lanes 8 to 13, 0.25 μg of purified primase was added to the DNA. **(B)** RPA stimulates primase binding to M13-ssDNA. 0.14 μg M13-ssDNA were incubated with no proteins (lanes 1 and 4) or increasing amounts (0.25 and 0.5 μg) of purified primase (lanes 2-3 and 6-7, respectively). In lanes 5 to 7, constant amounts (0.25 μg) of RPA were added to the DNA. After 1 hr. of incubation at RT the DNA and protein-DNA complexes in both gels were separated by native agarose gel electrophoresis. The DNA was then stained with Ethidium bromide. (w/o without).

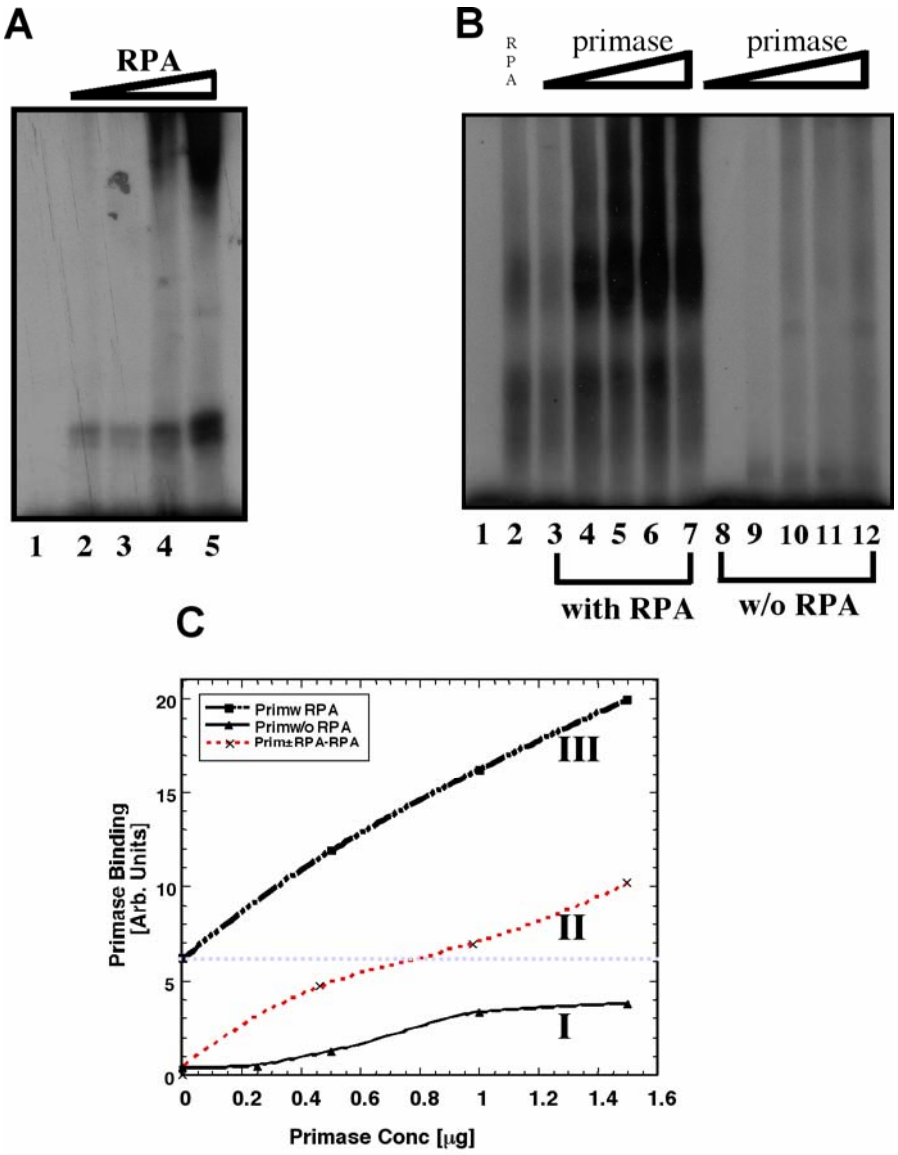

Figure 3.9: RPA stimulates primase (p48/p58) binding to DNA. (A) RPA binds to oligonucleotide oligo 58. Increasing amounts (0.25, 0.5, 1 and 2 μg) of RPA (lanes 2 to 5 respectively) were incubated with radioactively labeled oligonucleotide oligo 58. RPA forms with a homo oligonucleotide such as oligo dT_{30} a single complex, whereas RPA-oligonucleotide used here might have different conformation which show different mobilities in EMSA. **(B)** RPA stimulates primase (p48/p58) binding to oligonucleotide oligo 58. Increasing amounts (0.25, 0.5, 1, 1.5 and 3 μg) of primase (lanes 3 to 7 and similarly in lanes 8 to 12, respectively) were incubated with radioactively labeled oligonucleotide oligo 58. In addition, constant amounts (0.5 μg) of RPA were also added to the primase DNA binding assay (lanes 2-7). The protein-DNA complexes were separated by non-denaturing gel electrophoresis (6% acrylamide). Bound DNA was determined by autoradiography and quantified by phosphorimager. Only the shifted protein-DNA complexes are presented here since free DNA was over exposed. For comparison, the gel shift of oligonucleotide oligo 58 was also analyzed by non-denaturing gel electrophoresis and autoradiography in the presence of only assay buffer (lane 1) in both gels. **(C)** Quantification of primase and RPA co-operation. Radioactivity of gel in panel B was quantified and graphically represented in panel C. Line I shows only primase binding while line III shows primase binding in the presence of a fixed amount of RPA. Line II shows the difference of the binding activity between RPA alone and the combined activities of primase with a same and fixed amount of RPA as in lane 2 (6.2 arbitarary units). (w/o without).

decrease of mobility of the complex in the presence and absence of RPA (Figure 3.8B).

The results were highly significant and were further confirmed by replacing M13-ssDNA with oligonucleotide oligo 58 (Figure 3.9B). RPA binds efficiently with oligonucleotide oligo 58 (Figure 3.9A). In the experiment, a fixed amount of RPA was added to primase-DNA binding assay. As shown in Figure 3.9B, lanes 3-7 and 8-12 show primase binding with oligonucleotide oligo 58 but in lanes 3-7 a fixed amount of RPA was added, which significantly stimulated the primase binding to oligonucleotide oligo 58. This concentration of RPA also showed some interaction with the radioactively labeled oligonucleotide. Although the binding was significantly above background, the sum of the binding activities of the two proteins, primase and RPA incubated separately with DNA seems to be less than the DNA binding activity if both proteins were incubated with DNA together (compare lanes 9 to 12 plus lane 2 with lanes 4 to 7).

To verify this impression that RPA stimulated DNA binding of primase and that the binding of the two protein complexes RPA and primase were not simply additive, the radioactivity in the gel was quantified. The results are shown graphically in Figure 3.9C, in which line I shows the primase binding to oligonucleotide oligo 58 and line III shows the increase in binding in the presence of RPA while, line II shows the stimulation in binding. So, the quantification of the protein-DNA complexes shows that primase and RPA co-operate in their binding to DNA.

3.7 Iron effects primase binding to different oligonucleotides

Recently the group of HP. Nasheuer determined that primase dimer and the protein CTp58 contain iron in nearly a 1:1 molar ratio (B. Ashe, M. Görlach, R. Hilgenfeld, and H.P. Nasheuer, personal communication). In order to analyze whether iron has any influence on primase binding to DNA, shift assays were carried out with protein expressed in the presence of the iron-chelator, bipyridyl.

The amount of iron was also determined for the protein produced in the presence of bipyridyl. No iron above the limit of detection could be determined in these samples which suggest that the iron content of the protein was reduced by 90% or more (B. Ash and H.P. Nasheuer personal communication). For comparison, both protein samples "iron-containing" and "iron-free" were used for binding assay with oligonucleotide oligo 63 (Figure 3.10A). The comparison of the two forms demonstrates that binding is substantially reduced when iron-free protein (primase, p48/p58) was used in the binding assay.

Similarly, binding assay was also carried out for iron-containing and iron-free C-terminus of p58 (CTp58) proteins with oligonucleotides oligo 63 and oligo 58 as shown in Figures 3.10B and 3.10C, respectively. The proteins, which were isolated in the presence of iron chelator, bind several times less efficiently to oligonucleotides. This difference in binding suggests that iron plays a role in binding of both proteins (primase and CTp58) to DNA.

3.8 Addition of iron III to iron-depleted protein reconstitutes DNA-binding

In order to confirm that iron and not other elements play a role in primase to DNA binding, iron was added to binding assays of iron-depleted protein samples. In Figure 3.11, column 1 shows the negative control, representing the amount of radioactivity measured with only oligonucleotide oligo 58. The amount of DNA bound by untreated, iron-containing primase was set arbitrarily to 100% and is depicted in column 2, which serves as the positive control. In columns 3-5, iron-depleted protein was used and increasing amount of iron (Fe^{2+}) was added in lanes 4 and 5. The addition of Fe^{2+} to iron-depleted protein and oligonucleotide oligo 58 slightly increased the DNA binding activity of primase (compare columns 4 and 5 with 3). In contrast, as shown in columns 6 and 7, the addition of Fe^{3+} instead of Fe^{2+} to primase significantly increased its DNA binding activity. 800 μM of Fe^{3+} stimulated the DNA binding activity of iron-depleted protein by a factor of more than 3 and about 2/3 of the positive control was achieved (compare column

7 with 3 and 2, respectively). This figure clearly shows that binding of primase to DNA is stimulated by the addition of Fe^{3+} in iron-depleted samples (Figure 3.11). In contrast to this finding, addition of Mn and Mg did not stimulate binding of Iron-free primase (Malouli and Nasheuer, unpublished data).

3.9 Primase assay with iron-containing and iron-depleted primase

Primase activity using poly(dT) as template was carried out in the presence of oligonucleotide using primase containing iron or primase depleted of iron as an enzyme source. In the presence of competitor (oligonucleotide oligo 58), a significant difference in primase activity was observed between primase containing iron and iron-free primase. This confirms our results of iron requirement for primase for binding to DNA. The functional assay as presented in Figure 3.12 supports the binding studies. The iron-free primase, which has enzyme activity but has hardly any high affinity binding, is only moderately inhibited by oligonucleotide oligo 58. In contrast, the iron-containing primase dimer was readily inhibited by oligonucleotide oligo 58, which is effectively bound by the enzyme. This data underlines the influence of iron on the high affinity binding activity of primase.

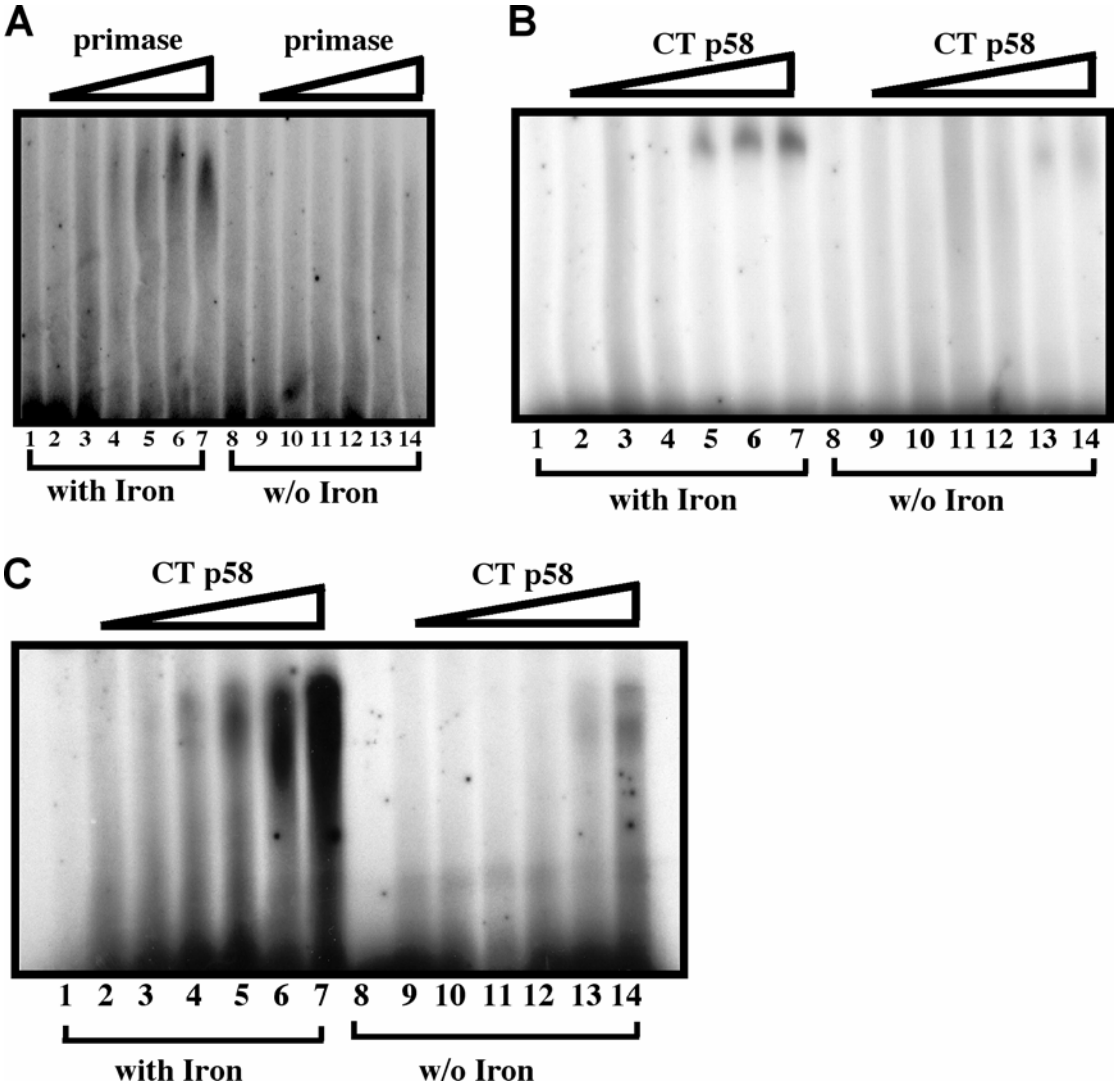

Figure 3.10: Effect of iron on primase binding to DNA. (A) Influence of iron on primase binding to oligonucleotide oligo 63. Increasing amounts (1, 2, 3, 4, 5 and 6 μg) of purified primase (p48/58) (lanes 2 to 7 and 9 to 14, respectively) were incubated with radioactively labeled olignucleotide oligo 63. In lanes 8 to 14, iron-free primase was used. **(B)** Iron influences C-terminus of p58 (CTp58) binding to oligonucleotide oligo 63. Increasing amounts (1, 2, 3, 4, 5 and 6 μg) of purified CTp58 (lanes 2 to 7 and 9 to 14, respectively) were incubated with radioactively labeled oligonucleotide oligo 63. In lanes 8 to 14, iron-free protein CTp58 was used. **(C)** Iron influences C-terminus of p58 (CTp58) binding to oligonucleotide oligo 58. Increasing amounts (1, 2, 3, 4, 5 and 6 μg) of purified CTp58 (lanes 2 to 7 and 9 to 14, respectively) were incubated with radioactively labeled oligonucleotide oligo 58. In lanes 8 to 14, iron-free protein CTp58 was used. The protein-DNA complexes were separated by non-denaturing gel electrophoresis (6% acrylamide). Bound and free DNA was determined by autoradiography. Only the shifted protein-DNA complexes are presented here since the free DNA was over-exposed. For comparison, the gel shift of oligonucleotide oligo 58 in gel "C" and oligonucleotide oligo 63 in gel A and B were also analyzed by non-denaturing gel electrophoresis and autoradiography in the presence of only assay buffer (lanes 1 and 8) in all three gels. (w/o without).

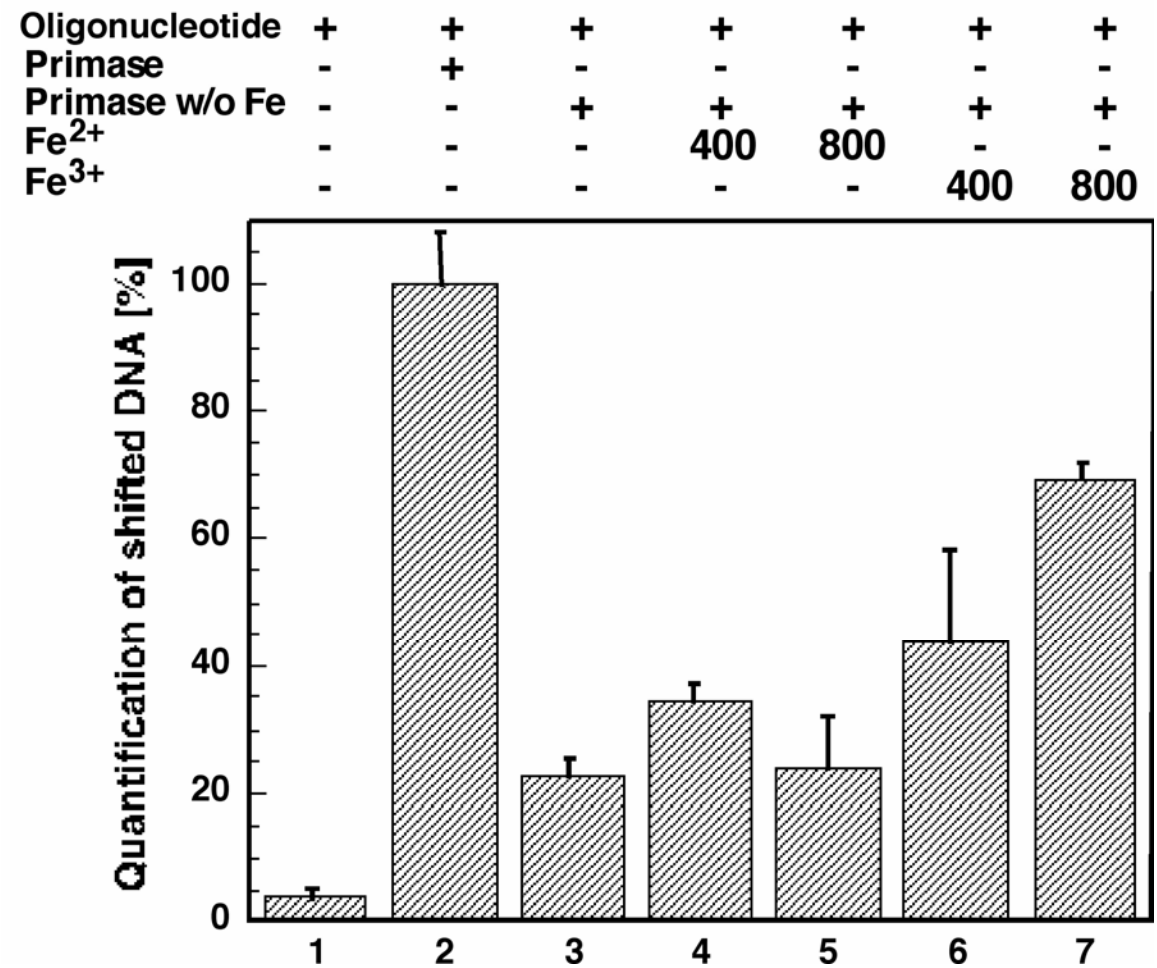

Figure 3.11: Addition of Fe^{3+} to iron-depleted protein stimulates binding of CTp58 with oligonucleotide. In order to find out whether iron can reactivate the binding ability in iron-depleted protein, Fe^{2+} and Fe^{3+} were added separately in binding assays of iron-free protein. Gels were quantified using phosphorimager. Column 1 is negative control, contains only oligonucleotide oligo 58 without protein, while column 2 is positive control showing shift with normal protein, which contains iron (arbitrarily set to 100%). Column 3 shows percentage of DNA shifted in binding assay with iron-depleted protein. Column 4 to 7 are similar to lane 3, with the only difference that in columns 4 and 5, Fe^{2+} was added at a concentration of 400 and 800 μM, respectively. In columns 6 and 7, Fe^{3+} was added to primase at a concentration of 400 and 800 μM, respectively.

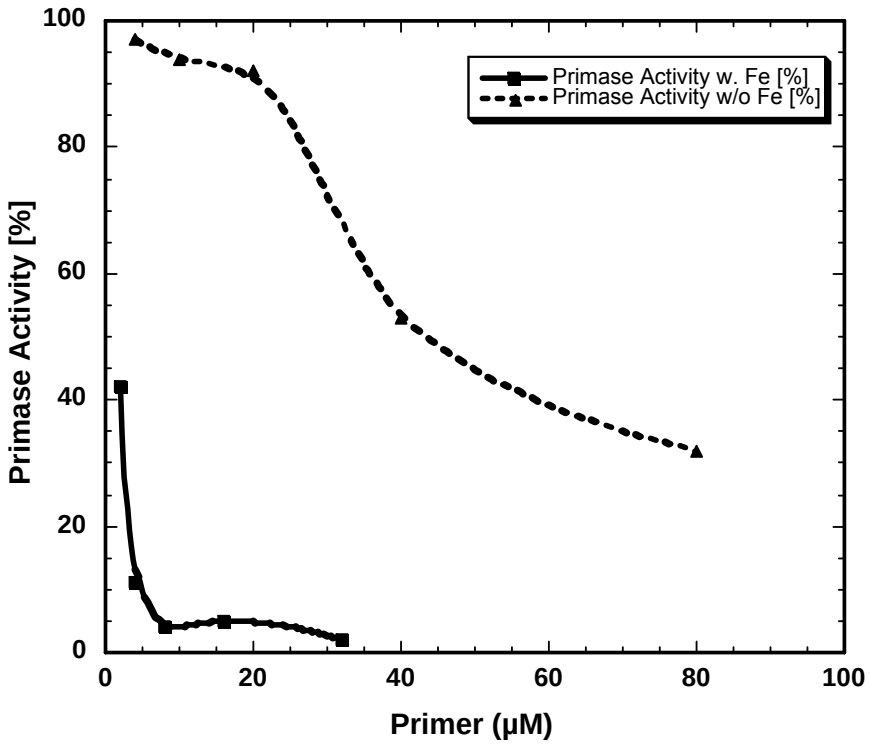

Figure 3.12: Inhibition of human primase activity. Primase activity using poly(dT) as template was carried out in the presence of oligonucleotide oligo 58 using primase containing iron or primase depleted of iron as an enzyme source. The Klenow DNA polymerase elongation assay was used and carried out in the presence of ^{32}P alpha dATP as described (Nasheuer & Grosse, 1988). Acid precipitated incorporation of radioactivity was determined by scintillation counting.

3.10 Localization of transfected (Pol α-GFP) fusion proteins in fixed and living cells

These studies of a newly discovered DNA binding activity of DNA polymerase α-primase led us to ask the question about the distribution and regulation of enzyme complex in human cells. In order to check their cellular localization, DNA polymerase α subunits p180 and p68 were cloned in pEGFP-C1 vector (Figure 2.1A) and expressed as GFP fusion proteins. These GFP-tagged DNA were

confirmed by sequencing and the protein expression was verified by western blotting (Figure 3.14 and data not shown). For localization experiments, GFP-tagged DNA polymerase α subunits p180 and p68 were transfected in logarithmically growing HEp2 cells. Cells were then further allowed to grow for 24 hrs. to allow expression of the transfected proteins. The strong signal derived from GFP enabled us to determine the sub-cellular distribution of fusion protein efficiently and conveniently. The results showed that both subunits were independently localized in the nucleus.

To analyze the distribution of p180 and p68 in living cells, similar experiment was carried out but instead of fixing, live cells were directly observed under microscope after 24 hrs. expression time. It can be seen from Figures 3.13A and 3.13B that there is no difference in localization. In both cases (fixed cells and in live cells) GFP-tagged Pol α protein localized in the nucleus. The transfection efficiency of the HEp2 cells with GFP-tagged DNA was almost 50 to 60% and the same amount of recombinant protein (1.5% over-expressed) was detected by westen blotting (data not shown).

3.11 Immunoprecipitation of recombinant p180 (T7-tag) in human cells with T7-tag antibody

In order to study the proteins interacting with DNA polymerase α, co-immunoprecipitation studies were carried out. The DNA polymerase α subunits p180 and p68 were cloned as T7-tagged fusion proteins in mammalian expression vector (Figure 2.1B). Expression of Pol α subunits in different mammalian cell lines was optimized (data not shown). Out of these cell lines, HEK 293 cells were selected on the basis of efficiency of transfection and high yields of expressed protein.

HEK 293 cells were transfected by pCMV-p180 (T7-tagged) clone. Proteins were immunoprecipitated with T7-tag antibody covalently attached to the agarose

beads and western blotting was done with p180 antibody (2CT25). Figure 3.14A, shows that T7-tag antibody pulled down T7-tagged p180 fusion protein, which proves that the transfected protein have good expression in cells.

3.12 Recombinant DNA polymerase α subunits form complex with each other

In order to determine whether ectopically expressed DNA polymerase α subunits associated with each other, HEK293 cells were transfected with DNA polymerase α subunits (T7 and GFP-tagged). HEK293 cells were transfected with p180 (T7-tagged) recombinant protein. Additionally, cells were co-transfected with GFP-tagged p68 as shown in lane 3 in Figure 3.14B. This co-immunoprecipitation revealed that recombinant T7-tagged, p180 protein, pulled down endogenous as well as extopically expressed p68 protein, which proves that transfected Pol α subunits form a complex with each other as well as with endogenous Pol α. In addition, these findings show that GFP-p68 can form a complex with the p180 subunit suggesting that the GFP fused to p68 does not diminish the association of p68 with p180.

3.13 Association of endogenous primase and ectopically expressed p180

To confirm that the recombinant proteins form a DNA polymerase α-primase complex, logarithmically growing HEK293 cells were transiently transfected with T7-tagged p180. It was tested whether the subunit p48 (primase) co-immunoprecipitated with p180 and the primase subunit was detected by western blotting with p48 antibody as shown in Figure 3.15A. Lane 1 is the negative control (only resin) and lane 2 contains crude extract from cells, which were not transfected, while lane 3 contains the crude extract from cells, which were transfected by T7-tagged p180. Western blotting was done with p48 antibody. Figure 3.15A demonstrates that p48 co-precipitates with p180 protein, which indicate that recombinant p180 and p48 form a complex in cycling human cells.

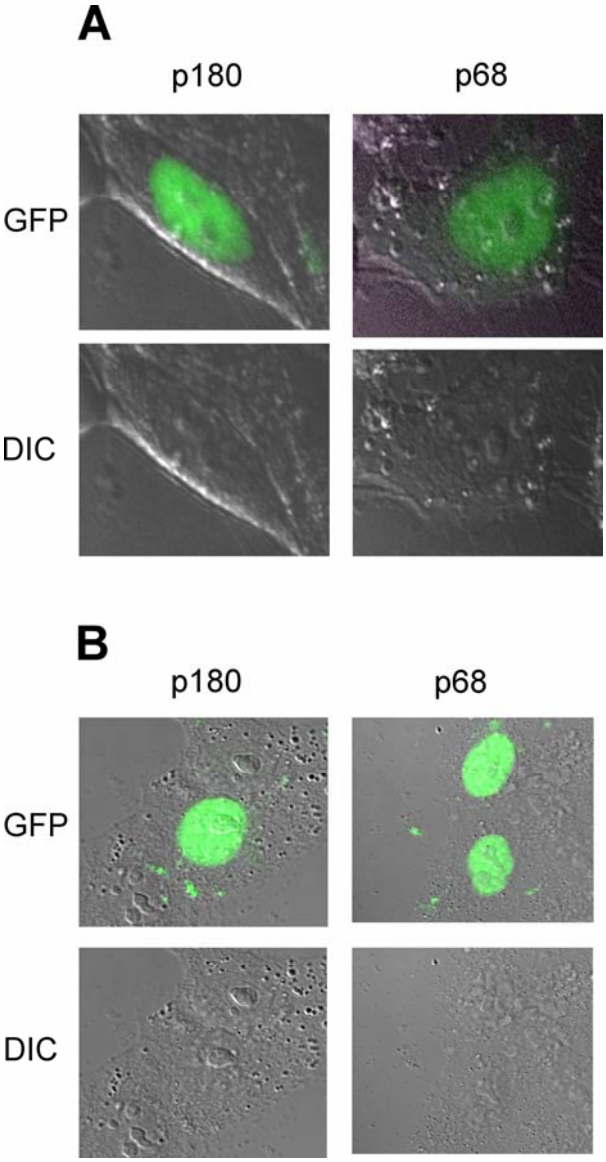

Figure 3.13: Localization of DNA polymerase α subunits (p180 and p68). (A) Microscopic images of the living HEp2 cells transfected with GFP fusion proteins. HEp2 cells were grown on 35 mm glass bottom microwell dishes and logarithmically growing cells were transiently transfected by GFP-tagged DNA polymerase α fusion proteins. After 24 hrs. cells were washed with PBS and directly analyzed under microscope. **(B)** Confocal laser scanning images of the HEp2 cells transfected with GFP fusion proteins and fixed by formaldehyde. HEp2 cells were grown on glass slides and logarithmically growing cells were transiently transfected by GFP-tagged polymerase α fusion proteins. After 24 hrs. cells were washed with PBS and fixed by 4% formaldehyde. Then slides were prepared as described in materials and methods and observed in microscope.

3.14 Association of replication protein A with DNA polymerase α-primase

During eukaryotic DNA replication the association of proteins is essential for the initiation and elongation steps. To determine whether the established vectors can be used to study these interactions, logarithmically growing HEK293 cells were transiently transfected with T7-tagged p68 while, p70 (RPA) co-immunoprecipitated and was detected by western blotting with p70 antibody, as shown in Figure 3.15B. In this figure, lane 1 is negative control (only resin) and lane 2 contains crude extract from cells, which were not transfected, while lane 3 contains the crude extract from cells, which were transfected by T7-tagged p68. The western blotting was developed with p70 RPA antibody. Figure 3.15B shows that p70 (RPA) co-precipitates with p68 protein, which indicates that p68 interacts with p70 (RPA) either directly or indirectly as part of a complex.

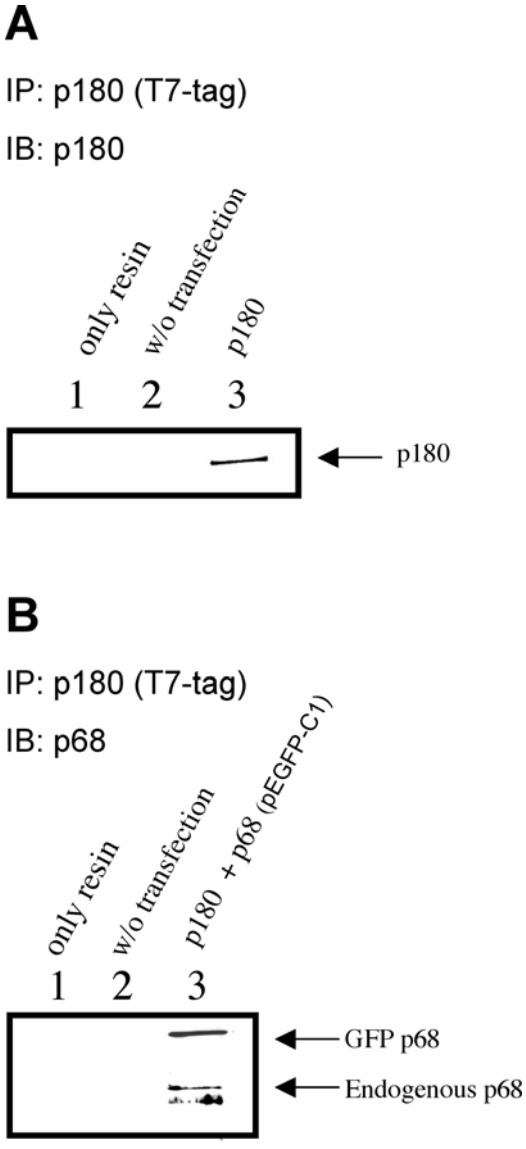

Figure 3.14: Immunoprecipitation of p180 (T7-tag) in human cells with T7-tag antibody. (A) HEK293 cells were transfected by T7-tagged p180 protein. After two days cells were lysed and protein concentration was measured by Bradford method. Then 500 µg protein for each sample incubated with 20 µl of resin (T7-tag antibody resin) with 500 µl IP buffer in cold room for two hrs. After washing, western blotting was carried out and the blot was developed with p180-specific monoclonal antibody (2CT25). Lane 1 was loaded only with resin. Lane 2 was loaded with the crude extract of the cells, which were not transfected. While lane 3 was carrying the crude extract from the cells, which were transfected by p180. The secondary antibody was HRP-conjugated anti-mouse, and the blot was developed with ECL. **(B)** Recombinant DNA polymerase α subunits form complex with each other. HEK293 cells were transfected by p180 subunit of Pol α. Additionally, cells were co-transfected with pol α second subunit p68 as GFP fusion protein. Lane1 was loaded only with resin. Lane 2 was loaded with the crude extract of the cells, which were not transfected. While lane 3 was carrying the crude extracts from the cells, which were transfected by p180. Additionally, cells in lane 5 were co-transfected with p68. 500 μg protein from each sample was incubated with 20 μl of resin (T7-tag antibody resin). The immunoprecipitated p68 subunit was detected by western blotting. Primary antibody for western blotting was polyclonal rabbit p68 antibody and secondary antibody was AP-conjugated anti rabbit antibody. The immunoblot was developed with NBT/BCIP. Endogenous and GFP-tagged p68 subunit are shown by arrows.

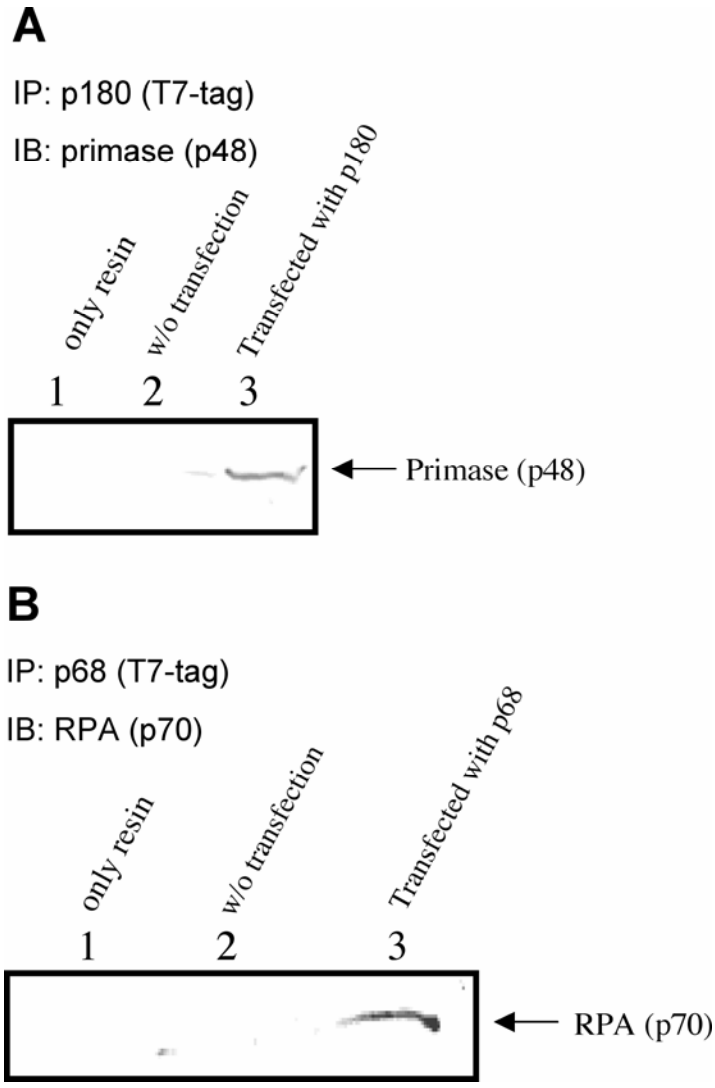

Figure 3.15: DNA Polymerase α subunits (p180, p68) co-IP with p48 and RPA (p70). Logarithmically growing HEK293 cells were transiently transfected by T7-tagged p180 (blot A) and T7-tagged p68 (blot B). The proteins co-immunoprecipitated with p180 and p68 were detected by western blotting. In both gels, lane 1 was loaded with resin, lane 2 was loaded with the crude extract of the cells, which were not transfected and lane 3 was carrying the crude extracts from the cells, which were transfected by p180 (gel A) and p68 (gel B). For blot A, primary antibody was polyclonal KAS2 for primase produced in rabbit and secondary antibody was AP-conjugated anti-rabbit antibody. While, primary antibodies for blot B was monoclonal rat p70 and secondary antibody was AP-conjugated anti-rat antibody. Both blots were developed with NBT/BCIP. Primase (p48) and RPA (p70) are shown by arrows. (w/o without).

4 Discussion

In eukaryotic cells, chromosomal DNA replication requires a large number of replication factors including three types of DNA polymerases, α, δ and ε. DNA polymerase α associates tightly with DNA primase, that synthesizes short oligoribonucleotide primers, which are then further elongated by its DNA polymerase activity. Therefore, the DNA polymerase α-primase complex is the only enzyme capable of initiating both leading strand synthesis as well as Okazaki fragment synthesis on the lagging strand (Hubscher et al., 2002). There is a significant sequence similarity between the p58 primase subunit and the 8 kDa domain of Pol β (Kirk and Kuchta, 1999). In Pol β this region is involved in DNA binding, which suggests a similar role for the primase large subunit. The Pol β-like region of p58 is located near the middle of p58 in the C-terminal half and may well comprise one of the regions of p58 that interact with the nucleic acid.

4.1 Expression and purification of recombinant human primase in *E. coli*

The initiation activity of primase is an essential function to start DNA replication *de novo*. The exact mechanism of initiation reaction is yet unknown. The p48 subunit alone can catalyze complete primer synthesis (Schneider et al., 1998). Relatively little is known about the role of the p58 subunit during primer synthesis (Copeland, 1997). It lacks detectable phosphodiester bond formation activity, although it greatly stabilizes the enzyme activity of the p48 subunit. The p58 subunit greatly enhances primer synthesis by p48 purified in the presence of a divalent cation and is essential for primer synthesis if no divalent cations were present during purification of the p48 (Schneider et al., 1998). Moreover, the role of p58 is to tether p48 to the 180 kDa subunit of Pol α (Copeland and Wang, 1993a). To study the functions of the primase subunits they were expressed using modified bacterial vectors that also allow the co-expression of protein complexes (Schneider et al., 1998). Proteolytically stable domains of human

primase were determined by partial tryptic digest of primase (Schlott and Nasheuer, unpublished data, summarized in Figure 3.1). The proteolysis products were then analyzed by SDS gel electrophoresis and peptide sequencing as described earlier (Pestryakov et al., 2003). The corresponding DNA was then cloned in *E. coli* vectors (Nasheuer, unpublished data). Since it is known that p58 binds to the DNA (Arezi et al., 1999), attempts were carried out whether the C-terminus of p58 binds to DNA. The cDNAs of dimer p48/p58 were fused to six histidine residues at their N terminus and expressed with bacterial expression vectors. These proteins were expressed and purified with high yields.

4.2 Characterization of primase binding to DNA

4.2.1 Primase binding to single stranded DNA (M13-ssDNA)

Electrophoretic mobility shift assay (EMSA) is a well-established assay to define the binding of proteins with different types of DNA. Therefore, in the present study EMSA was used to investigate the binding activity of primase with DNA. M13 is a single-stranded DNA (ssDNA) bacteriophage with a 6407-nucleotide genomic size. Binding experiments showed that primase dimer, p48 and the C-terminus of p58 interacts with M13-ssDNA (Figure 3.2). In the current study primase dimer and two components of it had such a high affinity that any cross-linking could be omitted. These binding results are consistent with the previously known binding assays by photo-cross-linking of p58 with ssDNA (Arezi et al., 1999). This apparent contradiction can be solved by the explanation that previous experiments were carried out with DNA that cannot form structures such as hairpins. This is consistent with our finding that oligo-pyrimidine and oligo-purine with lengths up to 80 bases cannot form a primase-DNA complex in EMSA. Moreover, oligonucleotides with a length of 37 nucleotides or shorter were also not efficiently bound in the EMSA and chemical cross-linking is required. A comparison of the DNA binding activity of primase and RPA with M13-ssDNA suggests that the primase-M13ssDNA complex is nearly as stable as that of RPA and M13ssDNA (K_D (primase dimer) = 0.5 nM and K_D (RPA) = 0.2

nM). In contrast to this result, the affinity of p48 and CTp58 is 6 times lower than that of primase dimer but still was easily detected by EMSA. These findings suggest that the DNA interaction domains of primase dimer are, similar to RPA, composed of various domains, which are also located on two subunits. The DNA binding activity of p48 is in agreement with its primase activity and that it binds to ssDNA cellulose during purification (Copeland and Wang, 1993b; Schneider et al., 1998).

This binding ability of p58 in conjunction with its location in the DNA Pol-prim complex suggests that p58 may be involved in the transfer of newly generated primer-template species from the primase active site to the Pol α active site. Photo-cross linking experiment (Arezi et al., 1999) of single-stranded DNA-primase complexes had revealed that only p58 subunit reacts with the DNA when photolysis was performed using the p48/p58 primase complex. This is very surprising, since p48 (i) contains the primase active site, (ii) can catalyze polymerization of ATP on oligo (A)-poly(dT) in the absence of p58 (Copeland and Wang, 1993a), (iii) can catalyze complete primer synthesis in the absence of p58 if purified in the presence of Mn^{2+} or Mg^{2+} (Schneider et al., 1998) and (iv) moreover, binds single-stranded DNA in the absence of p58 (Copeland and Wang, 1993a). This lack of reactivity of p48 in the cross-linking experiment carried out by (Arezi et al., 1999) suggests that in the context of the complex, DNA primarily binds to p58 or no residue of p48, that can be cross-linked, is in close vicinity to DNA. During primer synthesis, the DNA may move transiently into the catalytic site in p48, or alternatively, p58 may continuously bind the DNA and may "deliver" the template DNA to the active site in p48. Consistent with these possibilities, p58 greatly increases the extent of primer synthesis carried out by p48, which was purified with Mn^{2+} or Mg^{2+} (Schneider et al., 1998).

4.2.2 Primase binding with different structured oligonucleotides

To analyze the DNA binding of primase, a variety of oligonucleotides were designed to study which structure is preferentially bound by primase. Results show that primase (dimer, p48/p58), primase subunit p48 and p58 C-terminus bind to oligonucleotides namely oligo 58 and oligo 63 with high affinity. Primase did not show any significant binding to poly(dT), oligonucleotides oligo-pyrimidine and oligo-purine, the latter were each 80 nucleotides in length (data not shown). This is in contrast with the previous report of enzyme activity, where it shows a strong preference for deoxypyrimidine polymers (Badaracco et al., 1986). As, these long (80 nucleotides long) purine-rich or pyrimidine-rich oligonucleotides do not form any structure, this indicated that it is probably not the length but that some structures are probably involved in this high affinity DNA binding of primase. It was observed that primase dimer, p48 and the C-terminus of p58 (CTp58) bind very efficiently to oligonucleotide oligo 63 and a protein-DNA complex is readily detectable.

To further characterize this binding affinity, oligonucleotides that differ in their length and capability to form hairpin structures, were designed from oligonucleotide oligo 63, namely oligonucleotide oligo 21, oligo 29, oligo 36 and oligo 37. These four nucleotides form differently shaped structures as shown in Figure 3.3 (http://207.32.43.70/ biotools /oligocalc /oligocalc.asp). In comparison to oligonucleotide oligo 63, the binding of these shorter oligonucleotides was already impaired since in the absence of the cross-linking agent glutaraldehyde no detectable binding was observed with oligonucleotides oligo 21, oligo 29 and oligo 36. Moreover, very little binding was observed with oligonucleotide oligo 37 (data not shown). However, after the addition of a chemical cross-linking agent oligonucleotides oligo 29 and oligo 37 bind more strongly to CTp58 than to oligo 36 under these conditions that could possibly mimic the original situation when DNA polymerase α-primase binds DNA for RNA/DNA primer synthesis. Oligonucleotides oligo 29 and oligo 37 form hairpin structures (Figure 3.3). This indicates that binding simply does not depend alone upon the length of the

oligonucleotide but also on structure, because the oligonucleotides oligo 36 and oligo 37 are almost the same in size with the only difference that oligo 37 contains a stable hairpin structure. Moreover, the minimum DNA size requirement for pol-prim seems to be around 35-40 nucleotides long as minimum binding was only observed with oligonucleotide oligo 37 (37 nucleotides long). Our findings are comparable with those of RPA, which also shows weaker binding with short oligonucleotides, and it is known that hsRPA has strong oligonucleotides length dependence for binding. The occluded binding site for hsRPA has been defined as 30 nucleotides (nt) (Blackwell and Borowiec, 1994; Blackwell et al., 1996). To further study this binding behavior, primase assay was carried out in the presence of these oligonucleotides. The idea behind this experiment was to determine whether binding to oligonucleotides would interfere with primase activity. The oligonucleotides, which bind stronger to primase, compete for primase available for primase activity. The oligonucleotide bound with high affinity inhibited most efficiently the primase activity, while the weakly bound oligonucleotides were less effective. The data are consistent with this hypothesis and there is a good correlation between the stability of the primase-oligonucleotide complex and ability to inhibit primase activity.

4.3 Replication protein A (RPA) and primase co-operate in their binding to DNA

Eukaryotic RPA is a heterotrimeric single-stranded DNA (ssDNA)-binding protein that has multiple, critical roles in DNA metabolism (Iftode and Borowiec, 1997; Iftode et al., 1999; Wold, 1997). RPA participates in these diverse functions through its high affinity for ssDNA and its ability to interact with numerous proteins. Recently, it was found that RPA along with the DNA replication protein Cdc45p is involved in the recruitment of pol-prim at the chromosomal DNA replication origins (Walter and Newport, 2000; Zou and Stillman, 2000). Thus, both pol-prim and RPA are simultaneously present and likely to interact physically during initiation of DNA replication. This makes RPA a likely candidate

for the regulation of the catalytic activity of pol-prim. In the present work we were interested to know whether RPA and primase co-operate in their binding to DNA. It was found that in the presence of low concentrations of primase, the RPA-DNA binding reproducibly was 3 to 4 fold stimulated than in the absence of primase. Similarly, in the presence of low concentrations of RPA, the interaction of primase with DNA reproducibly was significantly stimulated (4-6 fold stimulation). Similar results were obtained with both, agarose and native polyacrylamide electrophoresis systems. Thus we propose that *in vivo*, RPA and pol-prim might be co-operating each other during binding to DNA and the assays used here are very simple ways to study physical and functional interactions of RPA and pol-prim. This interaction is important for: (1) Initiation of leading strand replication, (2) lagging strand replication, and (3) may be in signaling after DNA damage. Our results are consistent with the previously known reports, that RPA and primase show direct physical interaction (Weisshart et al., 2000; Weisshart et al., 2004) and RPA is necessary for activation of the pre-replication complex to form the initiation complex and for the ordered loading of essential initiator functions, e.g. the pol-prim complex to the origins of replication (Hubscher et al., 2002; Nasheuer et al., 2002).

4.4 Iron effects primase binding to DNA

During primase isolation it was observed that the protein shows a brown color. Using atomic absorption spectroscopy iron was found to be associated with primase (B. Ashe, M. Görlach, R. Hilgenfeld, and H.P. Nasheuer, personal communication). As it was predicted that metal binding might play a role in primase-DNA binding, the iron chelator, 2,2' bipyridyl, was added to the medium during protein expression to remove all iron from the medium and produce iron-free protein. Interestingly, binding of iron-depleted protein to DNA was substantially reduced. Binding of both, iron-depleted and iron-containing proteins were compared. The role of iron in DNA binding of primase was further confirmed by adding iron into the DNA binding assays with iron-depleted protein samples. It

was seen that binding recovers with addition of iron III to iron-depleted protein samples, which confirms the role of iron in primase binding.

Further, to confirm the iron requirement for primase-DNA binding, primase activity using poly(dT) as template was carried out in the presence of oligonucleotide oligo 58 with primase devoid of iron and iron-containing primase. A significant inhibition of primase activity was observed with iron-containing primase in the presence of oligonucleotide; a result confirming our previous binding results. But iron-free primase was only weakly inhibited at high oligonucleotide concentrations. With iron-containing protein, oligonucleotide traps the protein and reduces protein that can act as primase on poly(dT). On the other hand the iron-free primase is only able to bind to the oligonucleotide present in high concentration, whereas the unbound primase carries out the primase activity on poly(dT). These results also indicate that the iron is required for high affinity binding of primase but also that this high affinity binding site is not important for primase activity.

4.5 Localization of DNA polymerase α

DNA polymerase α-primase consists of four subunits and its subunit composition is highly conserved among eukaryotes. Previous studies (Mizuno et al., 1998) identified a putative nuclear localization signal for p180 (residues 1419 to 1437). It was also reported that association of p180 with p68 is important for both protein synthesis of p180 and translocation into the nucleus. However, they did not exclude the possibility that p68 might have a cryptic NLS of its own. The findings presented here do not agree with these results because when we transfected and expressed Pol α subunits, p180 and p68 separately in HEP2 cells, these proteins only localized in nucleus. These results were reproducible using GFP constructs in both cases, in living cells as well as in fixed cells indicating that localization is not caused by artificial manipulations such as cell

fixing and cells were not inadvertently selected from their particular appearance in the total population of randomly expressed cells.

4.6 Pol-prim interacting partners

Pol-prim interacts with a large number of proteins. The physiological significance of some of these interactions still needs to be confirmed (Stucki et al., 2001). Multiple interactions are expected for a protein complex playing a central role in several DNA transactions. Pol-prim is also involved in other relevant cellular processes, such as the response to DNA damage and the epigenetic control of chromatin structure, this indicates that the variety of functions played by pol-prim in many DNA transactions are far from being completely understood (Foiani et al., 1997).

To investigate the interacting partners of Pol α a new strategy was adopted. Pol-prim subunits were cloned and expressed as T7-tagged fusion proteins. Different proteins interacting with Pol α (T7-tag) were precipitated with T7-tag antibody covalently attached to agarose beads. This eliminated the chance of precipitating unspecific proteins interacting directly with Pol α antibody.

First of all, it was confirmed by western blotting (data not shown) and immunoprecipitation that recombinant proteins are expressed in human cells. Then, co-immunoprecipitation experiments proved that recombinant DNA polymerase subunits associated with the other subunits, endogenous as well as the recombinant co-expressed proteins. The replication protein RPA was found to interact with p68 subunit of DNA pol-prim, which led us to anticipate that the pol-prim and RPA complexes physically interact. The physical association of pol-prim and RPA support our findings that primase, as part of the pol-prim complex, functionally interacts with RPA in human cells.

5 Summary

DNA polymerase α-primase (pol-prim) is a four subunit complex (p180, p68, p58 and p48). Pol-prim is the only enzyme capable to start DNA synthesis *de novo*. It is evolutionary conserved from yeast to human and each subunit is essential for cell viability. The major role of pol-prim is the initiation of DNA replication at chromosomal origins and in the discontinuous synthesis of Okazaki fragments on the lagging strand of the replication fork. There is a sequence similarity between the p58 primase subunit and the 8 kDa ssDNA binding domain of Pol β. To determine whether p58 has any similar function, a proteolytically stable domain of human primase CTp58 (G266-S510), containing the Pol β-like region, was produced.

It was shown that primase (p48/p58) and its components p48 and CTp58 bind to M13-ssDNA. Furthermore, it was revealed that the CTp58 binds to oligonucleotides having different length and secondary structures. It was found that CTp58 binding not only depends upon the length but also on the structure of the oligonucleotide. This differential binding behavior with different structured oligonucleotides was further confirmed by primase assays. Moreover, we showed that RPA and primase co-operate in their binding to DNA. It was further found that iron is required for primase binding to DNA. Iron requirement was confirmed by a functional assay, using primase devoid of iron and iron-containing primase.

These studies of a newly discovered DNA binding activity of DNA polymerase α-primase led us to ask the question about the distribution and regulation of the enzyme complex in human cells. Therefore, GFP-tagged DNA polymerase α subunits p180 and p68 were analyzed in living as well as fixed cells. In both cases proteins were localized in the nucleus. Moreover, RPA was also found in a complex with DNA polymerase α-primase supporting the physical and functional interactions of DNA polymerase α-primase and primase.

Zusammenfassung

Die DNA-Polymerase-α-Primase (Pol-Prim) ist ein Vieruntereinheiten-Enzymkomplex bestehend aus p180, p68, p58 und p48. Pol-Prim ist das einzige Enzym, das die DNA-Synthese de novo beginnen kann. Sie ist von Hefe zu Mensch konserviert, und jede Untereinheit ist für die Lebensfähigkeit der eukaryotischen Zelle notwendig. Die Hauptrolle von Pol-Prim ist der Start der DNA-Replikation an chromosomalen Replikationursprüngen und der Beginn der Synthese von Okazaki-Fragmenten auf dem Folgestrang der Replikationgabel. Es gibt einige signifikante Ähnlichkeiten zwischen der Primase-Untereinheit p58 und der ssDNA-Bindedomäne von Pol □. Um festzustellen, ob p58 eine ähnliche Funktion in der proteolytisch stabilen C-terminalen Domäne CTp58 (G266-S510) wie Pol □ enthält, wurde CTp58 rekombinant hergestellt.

Es wurde gezeigt, daß das Primasedimer (p48/p58) und seine Bestandteile p48 und CTp58 M13-ssDNA binden. Außerdem wurde festgestellt, daß CTp58 an Oligonukleotide bindet, die unterschiedliche Länge und Sekundärstrukturen haben. Es wurde gefunden, daß die Bindung von CTp58 nicht nur von der Länge sondern auch von der Struktur des Oligonukleotides abhängt. Dieses Bindungsverhalten wurde durch Primaseaktivitätstests weiter bestätigt. Außerdem zeigten wir, daß RPA und Primase bei ihrer Bindung an DNA kooperieren. Es wurde weiter gefunden, daß Eisen für die Primase-DNA-Interaktionen erforderlich ist. Die Eisenabhängigkeit der DNA-Bindung wurde durch Primaseaktivitätstests untermauert, indem der Einfluß von Oligonukleotiden auf die Enzymaktivität von eisenfreiem und eisenhaltiger Primase getestet wurde.

Diese Studien zur neu entdeckten DNA-Bindungsaktivität von DNA-Polymerase-α-Primase warfen die Frage zur zellulären Verteilung von DNA-Polymerase-α-Primase in humanen Zellen und den Interaktionen des Enzymkomplexes auf. Mit Hilfe des fluoreszierenden Proteins GFP wurden die Fusionsproteine der

Untereinheiten p180 und p68 von DNA-Polymerase-α-Primase ausschließlich im Zellkern von lebenden und fixierten Zellen nachgewiesen. Ferner wurde gezeigt, dass DNA-Polymerase-α-Primase in Zellen mit RPA-Untereinheiten interagiert. Diese Untersuchungen unterstützen die Wichtigkeit der biochemischen Analysen zur physikalischen und funktionellen Interaktion von DNA-Polymerase-α-Primase, Primase und RPA.

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Selbständigkeitserklärung

I hereby declare that this thesis was composed by myself and that the work described is my own, unless otherwise stated.

Hiermit versichere ich, die vorliegende Arbeit selbständig und ohne fremde Hilfe verfaßt und keine anderen als die angegebenen Quellen und hilfsmittel verwendet zu haben.

Ferner versichere ich, daß ich diese Dissertation noch an keiner anderen Universität eingereicht habe, um ein Promotionsverfahren eröffnen zu lassen.

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